UNIVERSIDADE FEDERAL DE MINAS GERAIS
ESCOLA DE VETERINÁRIA
COLEGIADO DO CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

Ana Luiza Soares Fraiha

Swine Influenza Virus: Standardization of a RT-PCR and subtyping of field samples circulating in Brazil from 2012-2018

Belo Horizonte
2019

Ana Luiza Soares Fraiha

Swine Influenza Virus: Standardization of a RT-PCR and subtyping of field samples circulating in Brazil from 2012-2018

Dissertation submitted to the Graduate Program from Escola de Veterinária, Universidade Federal de Minas Gerais, in partial fulfillment of requirement of Master's degree in Veterinary Science.

Major: Preventive Veterinary Medicine
Advisor: Prof. Dr. Zélia Inês Portela Lobato
Co-advisers: Prof. Dr. Érica Azevedo Costa
Prof. Dr. Herman Sander Mansur

Belo Horizonte
2019

Fraiha, Ana Luiza Soares, 1991-
F812s Swine influenza vírus: Standartization of a RT-PCR and subtyping of field samples circulating in Brazil from 2012-2018 / Ana Luiza Soares Fraiha.
83. p: il.

Orientadora: Zélia Inês Portela Lobato
Coorientadores: Érica Azevedo Costa
Herman Sander Mansur
Dissertação apresentada à Universidade Federal de Minas Gerais, Escola de Veterinária.
Como requisito parcial de Mestre Ciências Veterinária
Inclui bibliografia

1. Sorologia - veterinária – Teses. 2. Suínos – doenças – Teses. 3. Vírus da influenza – Teses. I. Lobato, Zélia Inês Portela. II. Costa, Érica Azevedo. III. Mansur, Herman Sander
. IIII. Universidade Federal de Minas Gerais, Escola de Veterinária. IV. Titulo.

.

CDD – 636.408 96
Bibliotecário responsável Marcio Alves dos Santos CRB 3589/0
Biblioteca da Escola de Veterinária, Universidade Federal de Minas Gerais

É

UFMG UNIVERSIDADE FEDERAL DE MINAS GERAIS

FOLHA DE APROVAÇÃO

ANA LUIZA SOARES FRAIHA

Dissertação submetida à banca examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIA ANIMAL, como requisito para obtenção do grau de MESTRE em CIÊNCIA ANIMAL, área de concentração MEDICINA VETERINÁRIA PREVENTIVA .

Aprovada em 08 de Fevereiro de 2019, pela banca constituída pelos membros:

Profª. Zélia Inês Portela Lobato
Presidente - Orientador

Prof. Roberto Maurício Carvalho Guedes
Escola de Veterinária - UFMG

Drª. Beatriz Senra Alvares Da Silva Santos
Fundação Oswaldo Cruz - FIOCRUZ

Universidade Federal de Minas Gerais
Escola de Veterinária
Av. Antônio Carlos, 6627 -
Caixa Postal 567 – CEP 30123-970
Belo Horizonte – Minas Gerais
Telefone: (31) 3409-2057/2059(fax)
www.vet.ufmg.br
E-mail cap@vet.ufmg.br

Escola de Veterinária
UFMG

How priceless is your unfailing love, O God!

People take refuge in the shadow of your wings.

They feast on the abundance of your house;

you give them drink from your river of delights.

For with you is the fountain of life; in your light we see light.

Psalm 36:7-9

ACKNOWLEDGMENTS

To the Veterinarian School, for bestowing upon me one of the most valuable assets I now own: knowledge.

To my beloved parents, for their unconditional love, for having dedicated themselves to the complete human and intellectual development of their children. Thank you for daily teaching me to be the best person I can be, to have empathy for others, to have resilience in difficult situations, and to never lose faith. To my dear brother Pedro, for his love, care, friendship and for always being present in my life.

To my love, Vitor, for always making me happy! His genuine love and constant support complement my life and were crucial in my making it this far. Thank you so much, my dear!

To my beloved aunt Mercia, who constantly encourages me and teaches me to be strong in any circumstance without losing joy and hope. Thank your for your love, my dear aunt.

To my advisor, Professor Zelia Inês P. Lobato, for having taking me under her wing in LPVA and having believed in me. I thank you for all the opportunities you have offered me! Thank you for your valuable teachings, counsels and patience throughout this journey! Your dedication to the profession and your generosity to those around you are admirable qualities and are a great example to the school community and me.

To my co-advisor, Professor Erica Azevedo Costa, who did everything in her power to help me and when I didn't understand something she explained and taught me tirelessly. Your preoccupation with your student's learning is formidable. I am thankful for how you calmed me in moments of despair with inexhaustible patience. Thank you for being who you are! You are an extremely competent professional and a person with a kind heart who brings joy and laughter to everyone around you.

To my co-advisors, Professor Herman Mansur and Dr. Alexandra Mansur for introducing me to the fantastic world of nanotechnology. Thank you for welcoming me and helping in the engineering, it was enriching to be exposed to other areas of science.

To Professor Maria Isabel M. Guedes, thank you for the confidence and for the constant support in the laboratory! I admire your intelligence, your dedication and your sensitivity towards us! I am so thankful for your incentives, counsels, teachings and shared opportunities. Thank you!

To Dr. Ana Carolina D. Matos who is a unique and exemplary professional! You helped me with every millimeter of this scientific research. Suffered with me under the circumstances and thrilled with every discovery and success! Words fail to describe my gratitude for your mentorship, dedication and friendship! Thank you!

To Dr. Izabele Rehfeld, who received me as her undergraduate intern and through her mentorship I knew the scientific research, realizing its importance. You, Iza, are extremely competent in everything you do. You have taught me so much professionally and personally. I am thankful for your life and friendship!

To dear Grazielle Cossenzo, that is always willing to help me in anything I need and fills my days at the laboratory with laughter and good conversations! Thank you Grazi for your zel and support!

To the members of our laboratory: Aila, Alessandra, Barbara, Brenda, Fabinha, Kate, Marco, Mari, Stella, you are all amazing! It has been a pleasure to be with you on a daily basis learning and laughing.

To Cássia who motivates every one of her students to learn more about the scientific world. To Agda and Ana for all the help given.

To my great friends, Aninha, Bela, Bruna, Celina, Joilson, Lela, Lu, Pimps, Rapha, William. Thank you for all we have been through together in Vet. School! I'm thankful for how we have maintained a strong friendship. These two years of working on my masters would not have been the same without your presence at school and our delicious Gourmet meetings.

To my beloved and faithful friends: Angel, Grace, Gisele, Rafa, Amanda, Audrey and Dani! Thank you for always cheering for me, for your support in every moment, for understanding when I was absent and for your prayers and love. I am thankful for each one of your lives!

SUMMARY

LIST OF FIGURES

LIST OF TABLES

SUPLEMMENTARY

ABREVIATIONS

cDNA- complementary DNA
cH1N1- classical H1N1
DFA - direct immunofluorescent antibody testing
CPE – cytopathic effect
ELISA – enzyme-linked immunosorbent assay
FA - indirect immunofluorescent antibody testing
FAO – Food and Agriculture Organization
FluNet - Flu Epidemiological Surveillance Network
HA – hemaglutinin
HI – hemagglutination inhibition
H1N1pdm09 – 2009 pandemic H1N1 virus
IAV - influenza A virus
IFN-α – alpha interferon
IFN-β – beta interferon
IFN-γ – gamma interferon
IL1,2,3,4,6,8,13 – Interleukine 1,2,3,4,5,6,8,13
MDCK – Madin-Darby canine kidney
MDA - maternal derived antibody
MHC – major histocompatibility complex
mRNA - messenger RNA
NA – neuraminidase
NCBI - National Center for Biotechnology
NK cells - Natural Killer cells
NICs - National Influenza Centers
NP- viral nucleoprotein
NS1- non-structural protein
OIE – World Organisation for Animal Health
PA – acid polymerase
PAMPs - pathogens associated molecular patterns
PB1 – basic polymerase 1
PB2 – basic polymerase 2
PBS - Phosphate-buffered saline
PFU - plaque forming units
RNP – ribonucleoprotein particle
PRDC - porcine respiratory disease complex
PRRSV- reproductive and respiratory syndrome virus
PRRs – pathogen recognition receptors
RT- PCR – Reverse transcription polymerase chain reaction
sH1N1 – swine H1N1
SIV - swine influenza A virus
SN - Serum neutralization
SPF - Specific Pathogen Free
TNF- Tumor necrosis factor
TRIG - triple reassortant internal gene
VAERD – vaccine associated enhanced respiratory disease
vRNA - viral RNA
WHO - World Health Organization

Swine Influenza Virus: Standardization of a RT-PCR and subtyping of field samples circulating in Brazil from 2012-2018

Abstract

Influenza A virus (IAV) causes an acute respiratory disease in pigs, characterized by high morbidity in the herds. The IAVs have a segmented negative sense genomic RNA, which contributes to genetic mutations and virus rearrangements. The surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) are the main targets of host immune response, and mutations in these glycoproteins contribute to the genetic diversity of the virus. Three subtypes of swine influenza virus (SIV) circulate worldwide in the swine population: H1N1, H1N2 and H3N2. Rapid identification of viral subtypes is important for virus monitoring, assisting the detection of possible outbreaks and viral antigenic variations. The objective of this study was to standardize a RT-PCR test for the detection of SIV subtypes present in Brazil, analyzing their distribution combined with serological data from 2012-2018. The delineation of specific primers for the HA region was carried out from sequences of Brazilian samples, previously deposited at GenBank database. Characterized samples of H1N1pdm09, H3N2 and H1Delta were used as references. A first (One Step RT-PCR) and a second (Nested PCR) reaction was performed to standardize the test. Ninety-two field samples, previously positive for IAV, from 2012 to 2018 were used for the test validation. Sensitivity was assessed from the detection limit test and specificity from cross primer assays in control samples. The amplified PCR products from the field samples were sequenced and phylogenetic analysis was performed, as well as the p-distance calculation between samples of the same subtype. Partial HA sequences of H1pdm, from samples of the study and from other years, were translated and compared. Subtyping the N gene of H1Delta positive samples was performed. Serological analyzes were performed on 949 serum samples obtained from 2017-2018, evaluating the presence of antibodies against H1N1pdm09 and H3N2 by the Hemagglutination Inhibition (HI) test. The Nested test was more sensitive than the One-step reaction. There was no nonspecific cross amplification. Seventy-one (71/92-77%) of the field samples were subtyped by the Nested RT-PCR, 14 (14/71-20%) collected in 2012-2013, 35 (35/71- 49%) in 2014-2015, and 22 (22/71-31%) in 2017-2018. In 2012-2015, most of the samples were positive for H1N1pdm09, followed by H1Delta and H3N2, with H1N1pdm09 + H3N2 and H1N1pdm09 + H1Delta co-infection. In 2017-2018, there were more positive samples for H1Delta, followed by H1N1pdm and H3N2, and co-infection was also observed. From the H1Delta positive samples, only 57% (12/21) were subtyped for the N gene, with the majority positive for N2. A total of 716 serum samples were positive for IAV, and in 2017-2018 the occurrence of H3N2 was higher than H1N1pdm09 as well as the co-infection between H1N1pdm09+H3N2. By the phylogenetic analysis, partial sequences of each analyzed subtype have grouped into similar clusters. The p-distance analysis identified dissimilarity between samples into the H1Delta cluster and samples into H1N1pdm09 group. Amino acid alignment of H1N1pdm partial sequences, from different years, showed residual variations, especially at antigenic sites. The Nested RT-PCR technique was fast, sensitive and specific, which is recommended for SIV subtyping. Four subtypes of SIV circulate in Brazil. In 2012-2015 a higher occurrence of H1N1pdm was evidenced, but H3N2 was the most observed in 2017-2018. Analysis of amino acid residues of the H1N1pdm sequences demonstrated point substitutions between samples from different years, suggesting the evolution of the virus over time. Studies related to the genetic characterization of circulating SIVs in Brazil are essential to understand the evolution of viruses and their antigenic characteristics.

Keywords: Brazil, genotyping, Influenza A Virus, serology, swine

Resumo

O vírus influenza A (IAV) causa doença respiratória aguda em suínos, caracterizada por alta morbidade nos rebanhos. Os IAVs possuem RNA segmentado de senso negativo, contribuindo para mutações e rearranjos genéticos do vírus. As glicoproteínas hemaglutinina (HA) e neuraminidase (NA) são os principais alvos da resposta imune do hospedeiro, e mutações nas mesmas contribuem para a diversidade genética do IAV. Três subtipos do vírus da influenza suína (SIV) circulam mundialmente nos suídeos: H1N1, H1N2 e H3N2. A rápida identificação dos subtipos é importante para monitorar o SIV, auxiliando na detecção de possíveis surtos e variações antigênicas virais. O objetivo deste estudo foi padronizar um teste de RT-PCR para detectar subtipos de SIV presentes no Brasil, analisando suas distribuições combinada a dados sorológicos dos anos de 2012-2018. O delineamento de *primers* específicos para regiões de HA, foi realizado a partir de sequências de amostras brasileiras previamente depositadas no GenBank. Amostras caracterizadas de H1N1pdm09, H3N2 e H1Delta foram utilizadas como referências. Realizou-se uma primeira (*One Step* RT-PCR) e uma segunda (*Nested* PCR) reação para padronizar o teste. Noventa e duas amostras de campo, positivas para IAV, dos anos de 2012 a 2018, foram utilizadas para validar o RT-PCR. A sensibilidade foi avaliada a partir do teste de limite de detecção e a especificidade a partir de ensaios de *primers* cruzados nas amostras controle. Os produtos de PCR amplificados, a partir das amostras de campo, foram sequenciados, realizando-se a análise filogenética, bem como o cálculo do *p-distance* entre amostras de mesmo subtipo. Sequências parciais de HA, das amostras de H1pdm do estudo e de amostras de outros anos, foram traduzidas e comparadas. Subtipagem do gene N de amostras positivas para H1Delta foi executada. Realizou-se análises sorológicas em 949 amostras de soro obtidas nos anos de 2017-2018, avaliando a presença de anticorpos contra H1N1pdm09 e H3N2 pelo teste de Inibição da Hemaglutinação (HI). O teste *Nested* foi mais sensível que a reação *One-step*. Não houve amplificação cruzada inespecífica. Setenta e uma (71/92-77%) das amostras de campo foram subtipadas pelo *Nested* RT-PCR, sendo 14 (14/71-20%) coletadas em 2012-2013, 35 (35/71-49%) em 2014-2015, e 22 (22/71-31 %) em 2017-2018. Em 2012-2015, a maioria foram positivas para H1N1pdm09, seguido de H1Delta e H3N2, havendo também co-infecção de H1N1pdm09+H3N2 e H1N1pdm09+H1Delta. Em 2017-2018 observou-se mais amostras positivas para H1Delta, seguido de H1N1pdm e H3N2, sendo co-infecção também foi observada. Das amostras positivas para H1Delta, apenas 57% (12/21) foram subtipadas para o gene N, a maioria positiva para N2. Das amostras de soro analisadas, 716 foram positivas para IAV, e em 2017 e 2018 a ocorrência de H3N2 foi maior que a de H1N1pdm09 bem como a co-infecção por H1N1pdm09 e H3N2. Pela análise filogenética, sequências parciais de cada subtipo analisado se agruparam em grupos semelhantes. Na análise de *p-distance* identificou-se dissimilaridade entre as amostras de H1Delta e de H1N1pdm09 nos períodos analisados. O alinhamento de aminoácidos de sequências parciais de H1N1pdm de diferentes anos, mostraram variações residuais, especialmente em sítios antigênicos. A técnica de *Nested* RT-PCR mostrou-se rápida, sensível e específica, sendo recomendada para a subtipagem de SIV. Existe a circulação de quatro subtipos de SIV no Brasil. Nos anos de 2012-2015 uma maior ocorrência de H1N1pdm foi evidenciada, porém o H3N2 foi o mais observado em 2017-2018. A análise de resíduos de aminoácidos das sequências de H1N1pdm demonstrou substituições pontuais entre amostras de diferentes anos, sugerindo a evolução do vírus ao longo do tempo. Estudos relacionados à caracterização genética de SIVs circulantes no Brasil são essenciais para entender a evolução dos vírus e suas características antigênicas.

Palavras-chave: Brasil, genotipagem, Influenza A , sorologia, suínos

1. Introduction

Pork is the most consumed animal protein in the world, and Brazil is the fourth largest producer, China, the European Union and the United States, accounting for 3.39% of world production (Brazilian Association of Protein Animal - ABPA, 2017). The country is currently responsible for 8.90% of the pork volume exported worldwide, reaching almost US$ 1 billion/ year (ABPA,2017).

Despite the highly favorable data on pig production, the constant search to improve production has also brought several challenges, especially in the area of animal health. The breeding systems of pigs have evolved permanently and have led to the adoption of feedlot breeding methods, with increasing stocking densities and higher concentration of farms in certain geographic areas (Barcellos et al., 2008). These changes have risen the risk of the emergence of infectious diseases in swine farming, specifically in relation to respiratory diseases, since the economic losses are quite serious and fall on both the producers and the industry. These losses are a consequence of drug spending, reduced body development of affected animals, investments in prevention and control programs, mortality, as well as industry losses, through the condemnation of carcasses (Piffer and Brito, 1993; Maes et al., 2008).

Among the respiratory diseases that affect swine, swine influenza virus (SIV) is striking. SIV is an endemic zoonosis, affecting a wide variety of animal species and causing major economic problems affecting public and animal health (Brown, 2000). In swine, the disease is characterized by explosive outbreaks of respiratory disease, with high morbidity (reaching 100% of the herd) and typical symptomatology characterized by fever, respiratory distress, cough, nasal discharge and reduction in feed intake (Richt et al., 2003).

Swine Influenza virus (SIV) belongs to the species *Influenza A virus*, genus *Influenzavirus A,* family *Orthomyxoviridae* (ICTV, 2014). It has a single-stranded RNA genotype, negative sense, divided into eight segments that encode at least 10 viral proteins. The virus has a lipid envelope where the proteins haemagglutinin (HA), neuraminidase (NA) and matrix 2 (M2) are inserted, which protrude into the viral surface. The HA and NA surface glycoproteins are important for the pathogenesis and cycle of influenza virus multiplication and are the main targets of the host immune response (Cox et al., 2004). SIAVs are classified into subtypes according to the antigenic characteristics of their HA and NA molecules.

Because it has a segmented RNA genome, influenza A virus (IAV) presents a high index of genetic variability, generating new viral samples and / or subtypes, which can result in the occurrence of epidemics. Antigenic variability occurs through two distinct mechanisms, antigenic drift and genetic rearrangements (antigenic shift). The antigenic drift is characterized by minor changes in the genome, where point mutations mainly in the HA and NA coding segments select viral mutants that escape neutralization by antibodies generated either by infections either by previous vaccinations (Carrat and Flahaut, 2007). The antigenic shift occurs when two or more viruses infect the same cell and exchange segments of RNA, resulting in viral rearrangement. Viruses originating from viral rearrangements, if they are capable of being transmitted from individual to individual, have the potential to trigger pandemics, since they find a highly susceptible population due to the absence of previous immunity.

The swine has an important role in the epidemiology of influenza, since it can become infected with subtypes originating from different species such as human, avian and swine, allowing the emergence of new viruses with pandemic potential after the exchange of genomic segments of viruses originating from different species (Van Reeth and Ma, 2012). Due to this characteristic, it was then proposed that pigs can serve as a mixing vessel for human and avian influenza viruses, resulting in virus rearrangement with new combinations of swine, human and avian genes (Ma et al., 2009).

Three subtypes of swine influenza virus (SIV) are currently circulate worldwide in the swine population: H1N1, H1N2 and H3N2. And in Brazil the virus has been threatening the pork production since the emergence of 2009 pandemic influenza virus in humans, when several outbreaks associated to pandemic H1N1 (H1N1pdm09) virus were described in swine farms (Schaefer et al., 2011). Since then, H1N1pdm09 appears to have become endemic in Brazilian pig herds, with a high prevalence of positive animals. In addition to the H1N1 pandemic influenza virus, other strains and subtypes are present and widespread in Brazil, including H3N2, H1N2 and H1N1 subtypes of seasonal human origin (Dias et al., 2015; Ciacci-Zanella et al., 2015).

Vaccination is the most efficient method in the control and prevention of SIV, and the genetic and antigenic variation between circulating viruses in different regions and/or countries causes a variation in the composition of vaccines. Although vaccination is widely used, a good vaccine protection occurs when the HA vaccine protein is similar to the viral HA protein that

causes the infection (Van Reeth and Ma, 2012). In affected herds is important that the virus is quickly identified and subtyped since there is no cross-protection between heterologous subtypes.

Diagnostic tests should be always improved to have high sensitivity and specificity, be quickly performed and inexpensive. The application of the rapid and efficient diagnosis allows the monitoring of viral subtypes circulating in the swine population, thus obtaining necessary information for field veterinarians to apply efficient control measures in the farms.

2. Objectives

- To standardize a PCR technique to subtype H1N1, H1Delta and H3N2 from pig clinical samples positive for Influenza A.
- To compare partial sequences of H1N1pdm09 from 2012-2015 and 2017-2018 to Brazilian samples of pandemic SIV already deposited at Genbank.
- To analyze the distribution of subtypes of Influenza A using serological technique in routine diagnostic samples.

3. Literature Review

3.1. Etiology

Influenza viruses belong to the family Orthomixoviridae, which is composed of six genera: *Influenzavirus A, Influenzavirus B, Influenzavirus C, Influenzavirus D Thogotovirus, Quaranjavirus* and *Isavirus* (ICTV, 2014).

All influenza viruses are pleomorphic, enveloped with a negative-sense single-strand RNA with a segmented genome. Influenza A and B viruses contain eight RNA segments, while Influenza C and D viruses have only seven RNA segments (Krammer, 2018). The virus genera differentiate from each other based on the antigenic differences found in the nucleocapsid and matrix proteins (Cox et al., 2004). Influenza type A viruses can infect humans, birds, pigs, horses, aquatic animals, and bats, while influenza B and C viruses are found only in humans (Tong et al., 2013). Influenza type D was recently characterized and detected in pigs, but some

studies suggest that the virus from this genus plays a role in bovine respiratory disease (Flynn et al., 2018)

The eight RNA segments of IAV encodes 10-11 proteins (Figure 1). Segment 1, 2 and 3 (with 2341, 2341 and 2233 nucleotides, respectively), encode the PB2 (Basic polymerase 2), PB1 (basic polymerase 1) and PA (acid polymerase) proteins, respectively. In some strains of IAV segment 2 also encodes PB1-F2, which is associated with a pro-apoptotic activity. The PB2, PB1 and PA are part of the RNA polymerase complex, being that PB2 is important for the transcription of viral RNA (vRNA) acting in the mRNA cap recognition, and PB1 acts on the extension of vRNA. PA is related to vRNA replication (Bouvier and Palese, 2011). Segment 5 (1565 nucleotides) codes the viral nucleoprotein (NP) which is abundant and multiple copies of it conjugate with each genomic segment forming the ribonucleoprotein particle (RNP). The RNP is associated with the RNA polymerase complex and is related with transcription, replication and packaging of the genome (Portela and Digard, 2002). Coded by segment 7 (1027 nucleotides), the matrix protein M1 serves as an ion chanel and is the most abundant viral protein located beneath the lipid envelope that comes from the plasma membrane of the host cell. M1 plays an important structural role, conferring some rigidity on the structure of the virion. Moreover, the matrix protein M2 is also coded by segment 7, locating in the viral envelope. The matrix protein M2 is transmembrane protein that forms a proton channels that are important for viral cycle, contributing for RNA release into the host cytoplasm (Pielak and Chou, 2011). Moreover, segment 8 (890 nucleotides) codes the non-structural protein (NS1) and nuclear export (NEP) proteins. The NS1 plays an important role in transporting, assembling and translating RNA, whereas NEP (also refered to as non-structural protein 2, or NS2) is implicated in mediating the nuclear export of viral ribonucleoprotein (RNP) complexes (Webster et al., 1992).

The segment 4, with approximately 1778 nucleotides, encodes the hemagglutinin (HA) glycoprotein which has 550 amino acids, and the segment 7, with approximately 1413 nucleotides encodes the neuraminidase (NA) glycoprotein which has 454 amino acids. The glycoproteins HA and NA are the major surface proteins of influenza virus. The HA has an importance in the attachment and penetration of the virus into the host cell, since it binds to sialic receptors located in the cells of the respiratory tract, thus favoring the fusion of the viral envelope to the cellular membrane (Gambling and Skehel, 2010). It also has the property of agglutinating animal erythrocytes and contains the main epitopes that are targets of neutralizing

antibodies. The NA glycoprotein participate in the virions release from the host cell surface during viral release (Lamb, 2001) and are also a target of the host immune response. Because HA and NA are responsible for the binding to and release from host cell receptors, they are crucial in determining host specificity.

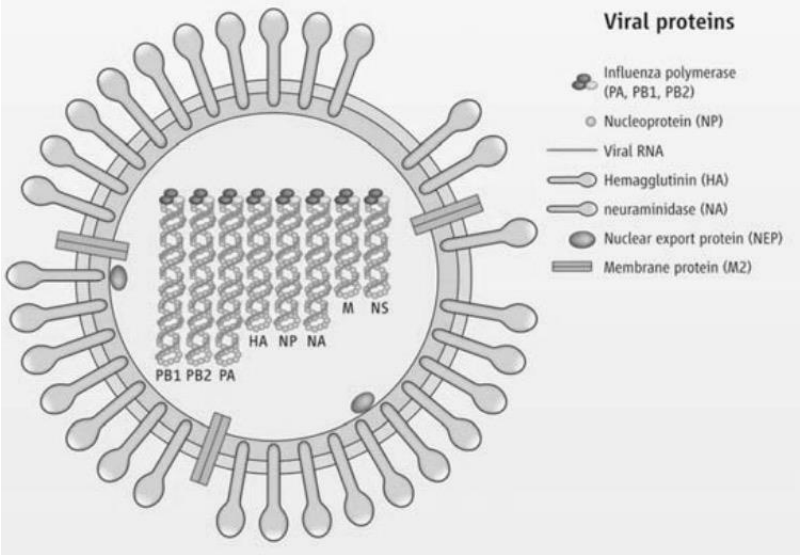

Figure 1. Structure of the Influenza A virus (Gambling and Skehel, 2010)

The HA and NA glycoproteins are the main targets of the host immune response being also highly susceptible to genetic variability (Cox et.al., 2004). Influenza A viruses are divided into subtypes based on the surface glycoproteins HA and NA. There are 18 different hemagglutinin subtypes (H1 through H18) and 11 different neuraminidase subtypes (N1 through N11) (Shao et al., 2017).

Neuraminidase glycoprotein removes sialic acid residues from cell-surface glycoproteins releasing progeny virus from the host cell. As a result, the new viruses infect new cells, spreading the infection (Bouvier and Palese, 2011). Antibodies against the NA, block the receptor-destroying activity, limiting the infection. The glycoprotein is a tetramer of identical subunits, and its polypeptide chain comprises 470 amino acid residues (Colman et al., 1993). Insertions and deletions can occur in amino acid sequences of different NA glycoprotein which varies the NA subtype. The enzyme active site is located at the center of each subunit, containing number of conserved charged amino acid residues (Gambling and Skehel, 2010), and this active site is a target for neuraminidase inhibitors such as antivirals.

SIV presents a high index of genetic variability, generating new viral samples and / or subtypes, which can result in the occurrence of epidemics. Antigenic variability occurs through two distinct mechanisms, antigenic drift and genetic rearrangements (antigenic shift) (Reid and Taubenberger, 1999).

The antigenic drift is characterized by minor changes in the genome, where point mutations mainly in the HA and NA coding segments select viral mutants that escape the neutralization by antibodies generated both by infections and by previous vaccinations (Carrat and Flahaut, 2007). It is well known that viral influenza RNA-polymerase represents the lack of proofreading function, leading to incorrect integration, deletion or insertion nucleotides during viral replication process resulting in viral mutation. Mutations at antigenic sites, specially at HA and NA glycoproteins contributes to the evolution of the virus, where new IAVs subtypes are able to escape from the immune protection of the host, since binding of antibodies to the virus do not occur (Shao et al., 2017). Amino acids substitutions in binding sites of HA also contribute for the change of host receptors preference (Landolt and Olsen, 2007). These changes contribute for cross-species transmission of influenza virus, because a virus that had preference for receptors found in a specific host, with the mutation could change the preference and infect another host.

The occurrence of antigenic shift is related to the segmented nature of IAVs genome and occurs when two or more viruses infect the same cell and exchange segments of RNA, resulting in viral rearrangement and new strains or subtypes. Viruses originating from viral rearrangements, if they are capable of being transmitted from individual to individual, have the potential to trigger pandemics, since they find a highly susceptible population due to the absence of previous immunity (Landolt and Olsen, 2007). The more recent human influenza pandemic was in 2009 due to a reassortant H1N1 influenza A virus with a genetic composition of genome segments from both North American triple reassortant swine virus origin and also Eurasian avian-like swine virus origin (Garten et.al, 2009). Swine have an important role in the antigenic shift, since it can be infected by avian and human IAVs (Shao et al., 2017).

3.2. Epidemiology

Influenza A virus affect a variable number of species including birds, dogs, horses, bats, swine and humans. The natural reservoirs of influenza viruses are the aquatic birds and some studies have suggested that bats may also be natural reservoirs of the virus (Tong et al. 2013).

In aquatic birds the virus replicates in the intestinal tract and have an HA binding preference for α2,3 sialic acid (SA) receptors on the host cell, while in humans the virus has a HA binding preference for α2,6 SA receptors, replicating in the respiratory tract (Yoon et al., 2014). Pigs are an important host in the influenza virus ecology since they present α2,3 and α2,6 sialic acid receptors in the respiratory tract, being susceptible to infection with both avian and human IAVs, acting as an intermediate host or ‘’mixing vessel’’(Brown, 2000), and are involved in interspecies transmission, facilitated by regular close contact with humans or birds. A study performed by Chothe et, al. (2017) demonstrated the co-expression of both avian and human type influenza receptors in the respiratory and gastrointestinal tract of little brown bats, which were compatible to avian and human IAV binding. These bats are widely found in North America evidencing the importance of the species for influenza epidemiology and emergence of zoonotic strains. Receptors for human and avian influenza viruses are also distributed throughout the respiratory tract of ferrets. This mammalian specie is highly susceptible to influenza viruses and has been used to study the pathogenesis and transmission of IAVs (Enkirch and Messling, 2015). Also, when experimental inoculated by intranasal route, these animals develop similar clinical signs of seasonal or avian influenza virus infection in humans, highlighting its use as animal model for studies (Maher et. al, 2004). The high susceptibility of the specie to influenza virus propitiates emergence of new viral strains and since these animals are in close contact with humans, there is an importance also for public health and in the epidemiology of the disease.

Swine influenza virus is endemic worldwide and outbreaks are characterized by a rapid onset of high fever, loss of appetite, labored abdominal breathing, coughing and weight loss, having a high morbidity, reaching almost 100% of the herd, and low mortality.

The incubation period of the disease in pigs ranges from 1-3 days, and 24 hours after infection pigs may excrete viruses and shedding can last for 7-10 days (OIE, 2015; Vincent et al., 2008). Recovery typically occurs between four to seven days post-infection (Van Reeth, 2007). Infected animals shed the virus by nasal secretions, and some studies have identified viral RNA and infectious viruses in feces, but the potential that SIV can be transmitted via the fecal route needs to be elucidated. Infected aquatic birds shed IAVs by feces, since the virus replicates in the epithelium of the intestinal tract. In humans shedding occurs mainly by nasal secretions. Gastrointestinal manifestation can occur, and the virus can also shed by feces (Kocer et al., 2013).

The SIV can be transmitted by contaminated aerosol, large droplets, direct contact with secretions of infected animals and indirect transmission between pigs and contaminated objects or surfaces. The major transmission route is the direct contact between a non – infected with secretions of an infected pig by the nasopharyngeal route (Brown, 2000). Clinical and subclinical manifestation contribute to the transmission of SIV in swine herds, highlighting the importance of control practices in the farms. The direct contact with contaminated water with bird feces has also been implicated as a source of avian origin IAVs, in swine farms (Ma et al., 2007). SIV is not transmitted by semen and no evidences of transplacental transmission has been reported. Indirect transmission occurs between pigs and contaminated objects or surfaces. The transmission between humans and pigs also occurs, highlighting the importance for routine surveillance of influenza in humans and pigs since contributes for a possible and early identification of novel influenza viruses with pandemic potential (Terebuh et al., 2010).

IAVs can survive for 24-48h on hands and nonporous surfaces, and 8-12h on porous surfaces (Bean et al.,1992). In aerosols IAVs can survive for several hours, depending on the relative humidity, as well as on ambient air temperature (Weber and Stilianakis, 2008)

Many risk factors are associated to the introduction and maintenance of SIV in the herds. At a farm level, the higher replacement rates and purchase of pigs from different regions, inadequate or absence of biosecurity measures, high animal density, large herd size, small distances between farms increases the number of contacts and the odds of spreading the disease within and between farms (Loeffen et al., 2009). Other animal species nearby or on the same farm, especially poultry and wild waterfowl constitutes a risk for the introduction of new influenza viruses in the herd. Humans are also source of IAVs, and vaccination of swine workers is extremely important to prevent the transmission of human IAVs to swine (Van Reeth and Ma, 2012).

In terms of seasonality, while in humans the seasonal influenza generally occurs during winter months, SIV outbreaks can occur during all seasons, especially in regions without temperature variation. Factors such as age of the pig, immune status, infection pressure, climatic conditions, herd management, replacement or entry of pigs from other herds and concomitant diseases may influence the number of cases of the disease (Van Reeth and Ma, 2012).

In a swine herd, outbreaks of clinical disease of SIV are more related to seronegative animals, which have no immune protection against the virus. And pigs of all ages may be

similarly affected by SIV, especially in farrow to finish farms being animals from farrowing, weaning and finishing phases frequently affected (Loeffen et al.,2009).

Sows and piglets play an important role in the epidemiology of SIV within the herd, especially in farrow to finish production systems. The sow's category has the higher number of seropositive animals, since it stayes longer periods in the herd having more chances of being infected during their life period (Loeffen et al. 2003), and the high rate of gilts replacement may contribute for the introduction of new viruses in the farm.

The sow placenta is epitheliochorial; thus, immunoglobulins are not transferred to the fetus during the intra-uterus life, and piglets receive high maternal antibodies titers by the colostrum ingestion. If the sow has been previously vaccinated or naturally exposed to SIAV infection, the maternal antibodies can prevent clinical disease in piglets, however the immune protection is partial, not preventing infection and reduction of the replication and elimination of the virus in these animals (Cox, et al., 2004). Therefore, piglets are highly susceptible to SIV and can be infected without manifestation of disease until some factors, such as stress or even the total decline of maternal antibodies, favors the appearance of clinical disease (Takemae et al., 2016). Suckling piglets with no clinical signs, especially in herds with inadequate immunity, are reservoirs of the disease contributing for the infection in weaned pigs and also maintenance of the virus in the herd (White et.al, 2017). Besides that, maternal antibody levels are variable in piglets from the same litter, thus susceptible piglets, with low antibodies titers, may be responsible for viral circulation in the farrowing house. Also, non-immune sows can be present during lactation, decreasing even more the protection level of antibodies acquired by the piglets (Loeffen et al., 2009).

The maternal antibodies level decrease along piglets age, and the lowest concentration was observed at 3-9 weeks and their absence at 9-10 weeks (Dias et al., 2015; Loeffen et al., 2003). In non-vaccinated and farrow to finishing herds with SIAV, it was demonstrated by Dias et al., (2015), that growing and finishing animals were seronegative for different subtypes of SIV. As there is a decrease of maternal antibodies, animals at the end of weaning and beginning of finishing phases are susceptible to infection. Another study performed in this same production system, showed that animals at the beginning of finishing phase were also highly associated with susceptibility to infection. However, this pattern was different in finishing herds, since the higher incidence of influenza virus infection was observed at the end of

finishing period, highlighting the importance of the pig flow in infection dynamic (Loeffen et al., 2009).

The evolution of influenza viruses is highly associated with the genome nature of the virus, the antigenic drift and shift associated with the occurrence of interspecies transmission, high transmissibility within a population, immune pressure and susceptibility of hosts (Brown, 2000; Shao et al., 2017).

The first case of SIV was observed in 1918 in the US when an outbreak of acute respiratory disease occurred in pigs (Koen, 1919). In the same year similar and severe outbreaks of respiratory disease were observed in humans, killing 20-40 million people worldwide. The episode in humans, namely Spanish influenza pandemic, was the first and worst influenza pandemic in history (Reid et al.,1999). Lately, studies have identified that the H1N1 influenza A virus was related to the outbreaks in humans and swine, and by sequencing analysis it was suggested that the virus have spread from humans to pigs, also because the disease in pigs was observed after its appearance in humans. (Shope, 1931; Reid et al., 1999)

SIV has evolved and disseminated worldwide since its first report, and the three subtypes H1N1, H3N2, H1N2 have been associated with disease in pig population, thus substantial diversity between the virus's subtypes circulating in different regions of the world (Vincent et al. 2014).

3.2.1. Swine influenza virus in North America

Following the first virus recognition in 1918, in the Midwestern U.S (Koen, 1919), SIV in North American swine population has become an important disease in the swine industry. The H1N1 virus derived from the Spanish flu was first isolated in 1930 (Shope,1931) and for almost 80 years this classical H1N1 (cH1N1) revealed genetic stability in the US (Easterday and Van Reeth, 1999; Vincent et al., 2014). In 1998 severe influenza-like illness outbreaks was observed in swine herds in North Carolina, Minnesota, Iowa and Texas. A double H3N2 reassortant virus containing gene segments (NS, NP, M, PB2 and PA) similar to the cH1N1 combined to segments (HA, NA, PB1) from a new human virus was isolated from North Carolina. This double reassortant virus did not spread efficiently among swine in the following years (Ma and Richt, 2010). However, a triple H3N2 reassortant virus containing gene segments from the classical swine virus (NS, NP, M), H3N2 human sazonal IAV (HA, NA, PB1) and an

avian IAV virus (PB2, PA) was isolated in the other states and widespread in the swine population. The genome composition of this new virus refers to a triple-reassortant internal gene (TRIG) cassette (Vincent et al., 2008) and in the North America the combination between the H3N2 TRIG and cH1N1 led to the emergence of new H1N2 and H1N1 subtypes (Webby et al., 2004). Nowadays, the vast majority of swine viruses contain the TRIG cassette combining with different subtypes of HA and NA. The subtypes H1N1 and H1N2 with HA and NA derived from human seasonal IAV and other genes from TRIG have emerged since 2005 and have spread across the U.S. in swine herds (Vincent et al., 2008). The HA from these human-like swine H1 viruses are genetically and antigenically distinct from those of classical swine-lineage H1 viruses (Vincent et al., 2014). Since the virus has evolved in North America, it was established a cluster classification based on the HA gene (Vincent et al., 2014). Viruses with the HA gene of the cH1N1 are classified into α, β and γ clusters and H1 viruses with HA genes most similar to human seasonal H1 are classified in δ cluster (Vincent et al., 2014). All H1 clusters can be found with the neuraminidases of the N1 or N2 subtypes (Vincent et al., 2014). Since the HA genes from the δ cluster may have emerged from introductions of human seasonal HAs from H1N2 and H1N1 the δ cluster differentiated phylogenetically into distinct sub-clusters, δ1 and δ2, respectively (Lorusso et al., 2011). The H3N2 SIV has also evolved leading to phylogenetic clusters I, II, III and IV and the cluster IV is also subdivided in emergent clusters from A to F (Olsen et al., 2006).

Until 2009 the H1 IAV evolved by drift and reassortment while maintaining the TRIG swine constellation. However, in 2009 a new pandemic occurred involving the subtype H1N1pdm09 (Lorusso et al., 2011) that was first detected in Canada in human population which rapidly spread worldwide being detected lately in swine species (Dawood et al., 2009). The internal gene constellation of this lineage is distinct from the H3N2- TRIG as the M gene is from Eurasian swine lineage, containing genes also from North American lineages (Garten et al., 2009). Since the 2009 pandemic, the level of endemic viral IAVs diversity is enhanced by interspecies transmissions events, and spillover events of H1N1pdm09, human seasonal H1 and H3 influenza viruses from humans to swine have been identified (Nelson et al., 2012). The interspecies introduction has led to reassortment events between H1N1pdm09 and swine IAVs, including the H3N2-TRIG increasing the diversity of circulating swine H3N2 genotypes since the introduction of H1N1pdm09 viruses in 2009, highlighting the importance of genetic surveillance for public and animal health (Ducatez et al., 2011).

3.2.2. Swine influenza virus in Europe and Asia

Swine influenza lineage viruses in Europe are different from the North American viruses. The cH1N1 viruses previously circulated in Europe, but it was in 1979 that an avian H1N1 started to circulate in swine population and lately a human H3N2 lineage from Asia emerged in Europe. These two lineages reassorted between 1983 and 1985 (De Jong et al., 1999). The H1N2 virus also emerged and spread in Europe and this lineage retained genetic characteristics of the reassortant H3N2 virus and HA gene from human H1N1 virus found in 1979 (Vincent et al.,2014). Studies have demonstrated that the Eurasian avian-like H1N1, human-like H3N2 and human-like H1N2 circulate in most of the Europe (Kyriakis et al., 2011). Other surveillance studies revealed the presence of H1N1pdm09 in some countries since the pandemic in humans (Welsh et al., 2010). Reassortants with the emergence of H1N1pdm09 have occurred such as a H1N1 virus in UK that acquired internal protein gene constellation of the H1N1pdm09, and HA and NA derived from swine endemic H1N2 viruses (Howard et al. 2011).

In Asia, since 1974 classical swine H1N1 viruses are enzootic in swine in China and co-circulated with H1N2 viruses with a N2 human origin. Human like H3N2 viruses were transmitted to pigs until the emergence of new human viruses (Peiris et al., 2001). Studies have shown that an European H3N2, introduced in 1999, have been circulating in southern China evidencing the direct introduction of swine influenza virus from Europe to Asia, very likely via pig movement (Zhu et al., 2011). In 1993, avian H1N1 viruses were also reported in China and had no Eurasian avian H1N1characteristics which may represent an independent interspecies transmission from the avian Asian reservoirs to swine (Guan et al., 1999). H1N1 reassortants between cH1N1 and European lineages were also detected in 2001 (Takemae et al., 2008). American triple reassortant H1N2 were regulary isolated from pigs since 2002 in China (Zhu et al., 2011), and this lineage and the triple reassortant H3N2 were also reported in Korea (Pascua et al., 2008). Since the 2009 H1N1 human pandemic, the virus has been repeatedly isolated from pigs in many Asian countries (Song et al., 2010) and most of these isolates resulted from different direct introductions from humans to pigs (Zhu et al., 2011)

3.2.3. Swine Influenza virus in Brazil

Influenza virus in pigs was first detected in Brazil in 1974, and the isolated virus was closely related to the classical North American swine virus A/swine/Illinois/1/63/H1N1 (Cunha

et al.,1978). Until 2009, few events related to clinical disease in pigs associated with SIAV were observed (Rajão et al., 2013a). Serological studies from sera collected between 1996-1999 revealed the presence of antibodies cross-reactive to swine and human H1N1 and human H3N2 in Brazilian pig herds (Brentano et al., 2002), evidencing that different subtypes of SIV were already circulating in the country. By 2006, a serologic survey performed in the state of São Paulo revealed a high prevalence of H1N1 and H3N2 (Mancini et al., 2006). Lately, another serological study has shown a prevalence of 20% for H3N2 in farms in the state of Paraná, and 46% of the analyzed farms were positive for SIAV (Caron et al., 2010). With the 2009 human influenza pandemic virus widespread, Brazilian swine population has experienced outbreaks with typical respiratory illness, resulting in an increase of surveillance studies that started to reveal additional influenza virus diversity in the country. The pandemic 2009 H1N1 virus was first isolated in Brazil between the years 2009-2010 in swine farms with respiratory signs, located in Santa Catarina and later the presence of the viruses was recognized in many other states (Rajão et al., 2013a). Previous to the H1N1 pandemic occurrence, antibodies against swine H1N1 and H3N2, and human H1N1 viruses were found in farms from Minas Gerais state, and sH1N1 was the most prevalent type (Rajão et al, 2013b). After the pandemic, another study performed in this same state has shown that the percentages of seropositive animals for H1N1pdm09 and H3N2 were 26.23% and 1.57%, respectively, and the percentages of positive herds for both viruses were 96.6% and 13.2%, respectively (Dias et al., 2015). Lately, serological studies performed in 2014-2015 in fifty swine herds from the South and Southeast of Brazil demonstrated that 94%, 68% and 18% of the herds presented antibodies against H1N1pdm09, H3N2 and H1N2 respectively, and co-infection between H1N1pdm09 and H3N2 was found in 36% of the herds (Fraiha et al., 2017). Most of the serological studies have shown that H1N1pdm09 is prevalent in Brazilian swine herds, but H3N2 and H1N2 are also present (Rajão et al., 2013a; Ciacci-Zanella et al.,2015).

In 2011, a reassortant human-like H1N2 derived from pandemic H1N1 was reported and detected during an outbreak of acute respiratory disease in a commercial pig herd and also in captive wild boars in Brazil (Biondo et al.,2014; Schaefer et al., 2015). Serological studies have shown that pig herds had been exposed to H1N2 influenza virus since 2010 (Ciacci-Zanella, 2012). Recently, a novel reassortant influenza A (H1N2), was recovered from a pig farm worker in southeast Brazil who had influenza-like illness. Genetic characterization has shown that the virus had a strong similarity to the H1N2 subtype virus detected in swine in

2011. This finding suggested a recent swine to human transmission from an H1N2 variant strain (Schaefer et al., 2015b; Resende et al., 2017).

Between 2009-2012, a phylogenetic study of SIV was conducted using nasal swabs and lungs samples collected from pigs showing typical clinical signs of influenza infection in different states of Brazil. From the sequenced samples, human seasonal H3N2 and H1N2 virus origin were detected and the analysis have shown that these subtypes were related to viruses that circulated in humans between 1990-2000. The matrix and some internal gene segments of the complete genomes sequences of the viral subtypes were related to H1N1pdm09. These findings suggested that human origin H3N2 and H1N1 have circulated for many years in Brazil, although not associated with clinical disease. However, after the introduction of H1N1pdm09, these subtypes were related to outbreaks of SIV in the country (Nelson et al., 2015). When analyzing these evolutionary findings with serological studies, it can be inferred that human origin H1N1 and H3N2 were circulating in Brazil before the 2009 pandemic, and the introduction of H1N1pdm09 to swine has favored the occurrence of reassortment events in swine (Nelson et al., 2015).

The dynamic epidemiology of Influenza virus in pigs highlight the importance of surveillance studies in Brazil not only in swine population but also in humans, since the human-to-swine transmission plays an important role in the epidemiology of the disease. And more studies about genetic characterization of SIAV should be performed, since genetic identification of circulating viruses in the country is extremely important to understand the evolution of the viruses and also to design effective swine influenza vaccines.

3.2.4. Human Influenza in Brazil

Currently, influenza A(H1N1) and A(H3N2) are the circulating seasonal influenza A virus subtypes circulating in human population. In addition, there are two distinct lineages of type B viruses that are also circulating as seasonal influenza virus. Great efforts are being made to monitor circulating viruses. The World Health Organization (WHO) initiated in 1947 the Flu Epidemiological Surveillance Network (FluNet) which is a global web-based tool for influenza virological surveillance. The networks received data from 140 National Influenza Centers (NICs) worldwide. Viruses harvested in various regions of the world are classified according to the antigenic type (A, B and C) and the influenza A virus in subtypes (H1N1, H3N2). The data is important for tracking the movement of viruses globally. According to the monitoring

and virus characterization the WHO recommends the composition of human influenza virus vaccine for each hemisphere for the following year. So then, since mutation of the virus is common, influenza vaccines are formulated each year in each country, according to the WHO recommendations. In Brazil, the influenza surveillance has been carried out by three national influenza laboratories: Instituto Adolfo Lutz (IAL), Oswaldo Cruz Foundation (FIOCRUZ) and Instituto Evandro Chagas (IEC).

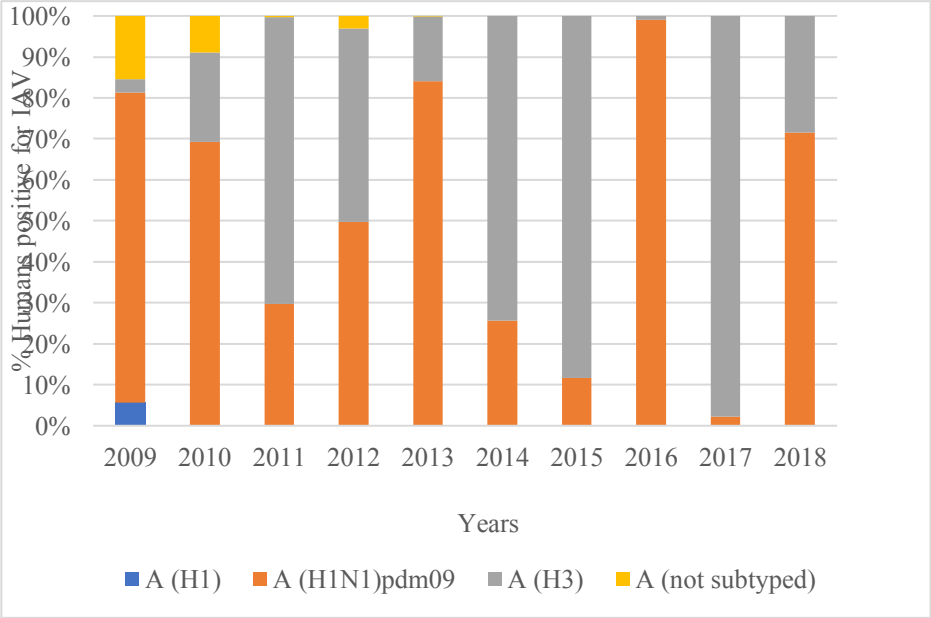

Figure 2. Percentage of occurrence of circulating Influenza A subtypes in the human population in Brazil between 2009-2018. Source: FluNet (www.who.int/flunet), Global Influenza Surveillance and Response System (GISRS).

According to data obtained by WHO FluNet, from the years 2009-2018 it was observed a great variation in serological data of subtypes H1N1pdm09 and H3N2 in the Brazilian human population (Figure 2). The predominance of one subtype relative to the other in different years and high cyclicity of them is notorious. The changes related to subtypes are closely linked to different implementations of vaccine strains in the vaccines each year and to the immune status of the population. Between 2011-2016 the recommended vaccines by WHO for the South Hemisphere contained in their composition the A/California/7/2009 (H1N1)pdm09-like virus, strains of H3N2 and Influenza B that have changed along this period. In 2016, genetic monitoring of IAV have demonstrated that the A (H1N1)pdm09 viruses, that were circulating in that year, were antigenically different from the vaccine virus A/California/7/2009. Therefore, the H1N1pdm09 vaccine strain recommended for 2017 changed for A/Michigan/45/2015

(H1N1) pdm09-like virus being also present in the composition a strain of H3N2 and Influenza B. In 2018, the population was vaccinated with a tetravalent vaccine containing the same sample of H1N1pdm09 and influenza B from 2017, but a different strain of H3N2 and a second and different influenza B strain was also added.

3.3. Pathogenesis

The natural infection by SIV occurs by inhalation of viral particles present in aerosols by direct or indirect contact. In the upper respiratory tract, inhaled particles deposit on the layer of mucus, and there are particles that reach the lower airways. Some experimental infections studies have demonstrated that SIV has a preference for the lower respiratory tract (Janke, 2013). The epithelial cells are the first host defense barrier, however, they are the primary target for IVs, as the virus strictly replicated in these cells present of both upper and lower respiratory tract. When reaching and replicating at the epithelial cells SIV destroys them and releases infectious viral particles to infect nearby cells (Flores et al., 2007). After inhalation of particles present in aerosols, the virus infects nasal cavity and trachea in the first 24-72h post-infection (PI), Epithelial cells in the bronchi and bronchioles are more extensively infected at 48-72h PI, while infection of alveolar epithelial cells tends to occur later in the course of infection, at 72-96h PI (Janke, 2013). It is supposed that depending of the amount of infectious particles that reaches the lower respiratory tract and lungs, the severity of the disease will be determined. The duration of infection and viral clearance is fast, thus, according to natural and experimental studies, it is possible to detect SIV in nasal swabs and lungs in the acute phase of disease, until seven days after infection (Brown et al., 1993).

Differences of severity and replication of different influenza subtypes have been observed in some studies. In an experimental infection study, the triple-reassortant H3N2 virus demonstrated higher levels of replication in tissues and shedding compared to a wholly human H3N2 virus (Landolt et al., 2003). Another study performed in Korea, showed that three groups infected with H1N2, H3N2 and H1N1 subtypes each, did not differ in the typically clinical manifestation of SIV. However, they differed in their gross lung lesions scores, since H3N2 infected pigs showed significantly higher lung lesions in the initial stage of infection compared to the other subtypes, and H1N1 subtype was associated with pulmonary lesions at a late stage of infection (Lyoo et al., 2014). The HA glycoproteins are responsible for the attachment of the virus in the sialic acid receptors in the cells, and it is possible that differences in amino acid in

the HA protein could let to differences in infectivity and replication of different viruses (Landolt et al., 2003).

SIV is generally considered to infect only the respiratory tract, but some studies have suggested that the virus may also reach other organs, since it was detected by direct diagnostic methods in mediastinal lymph nodes, spleen, ileum, colon and brainstem (De Vleeschauwer et al. 2009). Enterocytes are the main target cell of low pathogenic avian influenza virus (LPAI) and can also be infected by highly pathogenic (HPAI) strains in avian species. In these species the virus is mainly transmitted by the fecal-oral route and are excreted in feces, and clinical signs of diarrhea is common (Martins, 2012). In humans, the main route of infection by AIV is by the respiratory tract, but besides respiratory clinical signs influenza, virus infection can also infect the gastrointestinal tract causing severe symptoms such as vomiting, abdominal pain, anorexia, diarrhea (de Jong et.al, 2005)

With the epithelial cells impaired with SIV infection and replication, innate immune system starts to combat the infection with mucus and acute phase proteins. The infection in the respiratory epithelial cells leads to hyperemia, edema, necrosis, peeling and focal erosions of the epithelium (Crisci et al., 2013). Cell death by necrosis stimulates a strong inflammatory response through cytokine induction. The cytokines of the innate immune response contribute to the injury and destruction of the cells in the respiratory tract. The cytokine levels in the tissues are related to the severity of the infection (Janke, 2013). During acute phase, the cytokines such as IFN-α, TNF- α, IL-1, IL-6, IL-8 are found at the inflamed site and they also stimulate the recruitment of neutrophils, macrophages, dendritic cells and natural killer cells to prevent virus replication (Crisci et al., 2013). Cell death by SIV infection also occurs through apoptosis and if the virus does not stimulate apoptosis directly in an infected cell, it may do by the recruitment of leukocytes (Herold, 2008). Thus, the damage to the cells of the respiratory tract is mediated by virus infection and the cytokines produced by the innate immune response. Lately in the infection, specific immune response against the virus is modulated by T lymphocytes after the recognition of antigens.

3.4. Clinical signs and lesions

Clinical signs of SIAV generally starts within 1-3 days post infection in pigs. In the first days, pigs develop high fever demonstrated by hyperthermia (40.5 – 41.7°C), lethargy and anorexia (Janke, 2013). It may also be observed nasal and ocular discharge and conjunctivitis.

Moreover, when pigs are forced to move, dyspnea and tachypnea are also observed. Between 3-4 days post infection, pigs present a harsh deep barking cough, mainly due to bronchitis and bronquiolitis (Olsen et al., 2006). The major consequence of SIV is weight loss, with retarded growth and prolonged finishing time, which is related to the anorexia and inappetence (Kothalawala et al., 2006). Decreased semen production in boars and abortions in sows may also occur due to secondary effects of fever. Infertility, stillbirth and small litters are also noticed due to infection (Dee, 2015).

Animals recover within 5-7 days after disease onset. Typical outbreaks of influenza are limited to seronegative animals, without immune protection against the virus (Easterday e Van Reeth, 1999). It is important to highlight that subclinical infections occur frequently and are observed by elevated seroprevalence for viral subtypes even in the absence of significant respiratory disease. Animals with subclinical disease maintain the virus in the herd and, as they have sufficient immunity to prevent clinical disease, they can still be infected and eliminate the virus in the environment, predisposing the infection of naïve pigs or pigs with low virus immunity (Janke, 2013).

The occurrence of clinical disease in the animals is determined by diverse factors such as immune status, age, infection pressure, concomitant infections, climatic conditions and housing (Easterday and Van Reeth, 1999).

Clinical manifestation of SIV infection may vary depending on the immunity status of the herd. Acute outbreaks still occur, but infections are more frequently endemic with more attenuated clinical expression and co-infections with other respiratory pathogens. When the herd does not have adequate immunity against a variant of SIV, the acute clinical expression of the disease prevails, with a very rapid spread of the disease that can be found in animals of all ages presenting symptoms of high fever, anorexia, exertional expiratory dyspnea, and some animals may die. Recovery is rapid and infection is followed by very high levels of specific immunity (Janke, 2013).

When a variant of SIV is endemic in a swine population, clinical signs can be relatively predictable in given categories of a production system. Since nursering piglets have maternal antibodies against an endemic SIV, severe clinical signs cannot be relevant, but when these animals reach weaning age, clinical signs of SIV appear more significantly, since there is a decrease in maternal antibodies associated to a stress condition with the moving to a new facility

(Van Reeth and Ma, 2012; Janke, 2013). This situation is mainly observed when sow herd immunity is uniform, and pigs have roughly equivalent levels of passive protection, so clinical signs of SIV are more evident in older pigs when exposed to homologous virus and the maternal antibodies are not present (Janke, 2013). In farrow to finish herds, the frequently affected age groups are grow-finishing animals. Respiratory signs in sows are infrequent but when present it is demonstrated by reduced feed intake and fever (Loeffen et al., 2009).

Secondary bacterial infections can exacerbate the clinical signs of SIV increasing the severity and course of the disease. The SIV is also associated to the porcine respiratory disease complex (PRDC), combinated with pathogens, such as the reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae, Haemophilus spp, Pasteurella multocida*, and *Porcine circovirus type 2*, producing respiratory disease associated with high mortality (Brockmeier et al., 2002). Differences on clinical signs of SIV between different subtypes (H1N1, H3N2 and H1N2) have not been observed (Lyoo et al., 2014)

Since SIV enters the lung via airways, a cranioventral bronchopneumonia that affects a variable amount of the lung is a common macroscopic lesion found in infected animals (Janke,1998). Foci of consolidation are also evident in the hilar area and more dorsal portions of the cranial and middle lung lobes (Janke, 2013). Edema, foci of hemorrhages in the lung, mucopurulent exudates in the airways and edematous bronchial and mediastinal lymph nodes are also observed (Dee, 2015).

Histopathological alveolar atelectasis, interstitial pneumonia and emphysema, necrosis of bronchial and bronchiolar epithelial cells as well as accumulation of necrotic debris and neutrophils in the bronchial and bronchiolar lumina are observed in the acute phase of the disease. In a late stage, hyperplastic or nearly normal epithelium characterize the phase of repair. Mononuclear cells are also present, and regions of fibrosis may be observed (Janke, 2013).

3.5. Immune Response

Upon IAV infection, innate and adaptive immune response acts to defend the host. The innate immune response is the first line of defense against viral infection, which acts preventing infection of the respiratory epithelium and control virus replication. When cells are infected by IAVs, pathogens associated molecular patterns (PAMPs) are recognized by the host pathogen

recognition receptors (PRRs) such as TLR7, TLR3 and RIG-I. With this recognition, pro inflammatory cytokines and interferons are produced, such as IL6, IL1, TNF, IFN-α, IFN-β, contributing for the recruitment of innate cells, such as NK cells, monocytes, neutrophils, dendritic cells and also macrophages. The produced interferons act as antiviral to efficiently limit replication and spread of the virus by inhibiting protein synthesis in host cells (Killip et al., 2015). Interferon also stimulates dendritic cells enhancing antigen presentation to CD4+ and CD8+ T cells, contributing for the beginning of the adaptive immune responses. Macrophages are important to phagocyte influenza virus-infected cells, but also are responsible to induce influenza virus pathology, since when activated in the lungs, induce the production of nitric oxide synthase 2 and tumor necrosis factor α (TNF α), which led to tissue damage. Natural killer cells recognize antibodies bound to influenza virus infected cells and lyse these cells. The dendritic cells (DCs) contributes to the beginning of the adaptive immune system, which is the second line of defense against IAVs infection. DCs present the antigen to T CD8+ lymphocytes by the major histocompatibility complex (MHC) class I molecule (Chen, 2018). The CD8+ T cells differentiate into cytotoxic T lymphocytes, which produces cytokines to eliminate virus-infected cells, preventing the production of progeny viruses. Viral proteins can also be presented by the MHC class II for the recognition of CD4+ T helper (Th) cells, which differentiates into Th1 and Th2 CD4+ T cells. Th1 cells express antiviral cytokines, such as IL-2 and IFN-γ and TNF, that regulate CD8+ T cells differentiation and activate alveolar macrophages. Th2 cells produces IL-4 and IL-13 that may lead to B cell proliferation and maturation to antibody producing plasma cells (Chen, 2018).

Influenza virus infection induces virus specific antibodies response. Specific antibodies against the two surface glycoproteins HA and NA are related to protective immunity (Potter and Oxford, 1979). The HA specific antibodies bind to the globular head of the HA, inhibiting virus attachment and entry in the host cell. The antibodies against HA are considered to be the most important as they can neutralize the virus, preventing the disease. The binding also facilitates phagocytosis and destruction by natural killer cells. Although antibodies against the neuraminidase are less effective in the prevention of the disease, they also have a protective function, as limits virus spreading, facilitates the action of natural killer cells and also contributes for the clearance of virus infected cells (Cox et al. 2004; Kreijtz et al., 2011).

The main antibodies isotypes found in the IAV specific immune response are IgA, IgM and IgG (Charley et al., 2006). IgA is the primary antibody detected at the mucosal surface post

infection, contributing for mucosal protection removing the agent before it infects the cells. The local IgA response stimulated by natural infection lasts for at least 3-5 months (Cox et al., 2004) and since is rapidly produced after IAV infection, the presence of IgA can be an indicative for a recent influenza virus infection (Voeten et al., 1999). Intranasal vaccine administration induces both IgA and IgG production, differently from parenteral administration that induces only IgG (Chen, 2012). In serum, the three major Ig classes can be detected within 10-14 days post-infection, as IgM levels peak after 2 weeks and IgG at 4-6 weeks. The higher the titers of antibodies against HA, the higher the immune protection. Serum anti-HA are the most commonly measured correlate of protection against SIV, and a HI titer higher or equal to 40 is indicative of protection (Cox et al., 2004). Maternal antibodies, subtype IgG, protects piglets from clinical disease, but do not prevent infection and viral transmission. Thus, infected suckling piglets play an important role for the maintenance of the disease in the herd, since it may have subclinical infection (Loeffen et al., 2003).

After a primary infection with SIV, it is induced a protection against reinfection with the same or a very similar virus strain, however, when animals are re-infected with heterologous viruses, partial protection is provided (Vincent et al., 2008). Broad protection for many different SIV subtypes is a challenge for vaccine production, since the virus constantly changes through mutation or reassortment. The HA glycoprotein is the main target of the humoral response mediated by antibodies induced by infection. The antigenic drift, which point mutations occur in antigenic regions of the HA, and the antigenic shift, which raises novel subtypes of IAVs, alters antigenicity of the viruses limiting the antibodies recognition which contributes for virus escape from the humoral immunity (Kreijtz et al., 2011).

The HA glycoprotein is synthesized in ribosomes, from a homo-trimer precursor protein HA0. At the end of the virus replicative cycle, during the transport of the glycoproteins to the plasmatic membrane, this molecule is cleaved by cellular proteases into HA1 and HA2. The HA1 (327 amino acids) and HA2 (222 amino acids) are disulfide linked and each monomer of the HA molecule consists of a globular head domain and a stem domain. At the globular domain is located the most part of HA1 and at the stem domain parts of HA1 and HA2 (Bouvier and Palese, 2011; Sriwilaijaroen and Suzuki, 2012). Five antigenic sites were identified at the HA monomer: Sa, Sb, Ca1, Ca2, Cb which are mostly encountered at the HA1 subunit (Figure 3). The antigenic sites Sa (residues 128-129, 156-160, 162-167) and Sb (187-198) are located in the upper and back region of the globular head of HA1 subunit, respectively. The Ca antigenic

site have subsites denominated Ca1 (residues 169–173, 206–208, 238–240) and Ca2 (residues 140–145, 224– 225), which are between adjacent HA monomers. The Cb (residues 74–79) antigenic site is located at the bottom of the globular head of HA1 (Sriwilaijaroen and Suzuki, 2012).

Substitutions of amino acids at these antigenic sites during infection are associated with antigenic change being also a mechanism to escape the host immune response (Gambling and Skehel, 2010). Monitoring these changes is important to understand the virus evolution and selection of vaccine strains. The HA1 subunit, also carries receptors binding site, (residues 99; 133-139; 152-154), being responsible for attachment of the virus to specific sialic sugar chains on the host cell surface (Sriwilaijaroen and Suzuki, 2012). Depending on the receptor specificity the binding will occurs on α2,3 receptors in the intestinal tract of avian, or α2,6 receptor present in the respiratory tract cell in humans. There is also binding of both α2,3 and α2,6 receptors in the respiratory tract cells in pigs. Changes in the amino acid residues in the receptor binding region of HA can cause alteration of influenza binding preference, predisposing the occurrence of pandemics (Sriwilaijaroen and Suzuki, 2012). After the binding of HA1 to sialic acid receptors, occurs endocytosis of the influenza virus which is taken up into an endosome. The low pH at the endosome changes the conformation of HA2 which mediates the fusion of the host endosomal membrane with the viral membrane, allowing entry of viral ribonucleoprotein into the host cell (Bouvier and Palese, 2011). Other important sites that are found in both HA1 and HA2, are the glycosylation sites. At the globular domain of the HA1 there are more variable glycosylation sites. These sites are important for the stability of the HA structure, especially those at the HA2 domain (Mukherjee et al., 2015). They are also important to escape from host immune response since it mask regions susceptible to antibody neutralization. When there is glycan variability and acquirement of additional glycosylation sites HA became more able to escape the host immunity (Mukherjee et al., 2015).

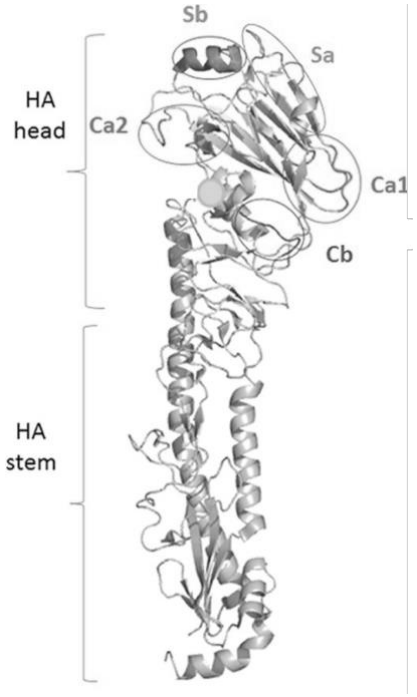

Figure 3 - Diagram of an uncleaved hemagglutinin monomer from the 1918 influenza A virus (H1N1). The head contains five predicted antigenic sites (Sa, Sb, Ca1, Ca2, and Cb). (Figure adapted from Sum,2013.)

3.6. Diagnostics

Outbreaks of acute respiratory disease in swine, involving a large number of animals, must necessarily be investigated for influenza. The collection of samples, as well as their packaging and application of accurate and rapid diagnostic tests are essential for virus detection in order to contain outbreaks, perform epidemiological surveillance, control and prevent infection.

The process of influenza diagnosis begins with the correct sampling, and animals that are in the acute phase (presenting fever, cough, nasal discharge) have to be preferably selected (Janke, 2000). For virologic and molecular diagnostic tests, samples of nasal discharge, lung and oral fluid must be collected and kept refrigerated (4^{o}C) if processed until 48 hours post collection, otherwise must be conditioned at -80^{o}C in case the samples are stored for a long time (OIE, 2015). For histopathological and immunohistochemistry analysis, lung is essential; for serological tests, blood serum and oral fluid can be collected and kept refrigerated. Oral fluid has been used also for the detection of virus, and some studies have demonstrated that the method is sensible (Detmer et al. 2011).

Virus and antibodies detection for SIV infection are commonly performed by different diagnostic methods such as viral isolation and molecular tests for nucleic acid detection (conventional and real-time RT-PCR), hemagglutination inhibition (HI), enzyme-linked immunosorbent assay (ELISA), indirect and direct immunofluorescence and serum neutralization (SN).

Virus isolation can be conducted preferably in Madin-Darby canine kidney (MDCK) cells, which is permissible to various subtypes and strains of SIAVs, but swine primary cells susceptible to SIV can also be used (OIE, 2015). The inoculated cells are periodically examined for characteristic cytopathic effect (CPE). Embryonated SPF (Specific Pathogen Free) eggs are also used for isolation and demonstrates better efficient replication of certain virus samples inducing higher viral titers (Hussain et al., 2010).

The use of molecular techniques to directly detect virus in samples from animals, facilitates the rapid identification and genetic characterization of influenza A viruses. Polymerase Chain Reaction (PCR) is a powerful technique for the identification of influenza virus, since it is specific and sensitive, detecting nucleic acids present at very low levels in clinical samples. The viral genome of IAVs is a single-stranded RNA, being necessary to synthetize a DNA copy (cDNA) prior to the PCR reaction, and for this, a reverse transcriptase (RT) polymerase is used (WHO, 2011).

The choice of primers sequence for the detection of Influenza A is usually based on the M protein, which is conserved among the IAVs subtypes. Specific primers pairs for the hemagglutinin (HA) gene are designed on known sequences, mainly from currently influenza circulating viruses (Zhang and Evans, 1991; OIE, 2015). Amplicons produced in PCR reactions can be sequenced determining the subtype of the virus, by comparing with sequences that are already deposited in databases such as Genbank. Conventional RT-PCR is used to detect the presence or absence of certain genomic fragments, while quantitative RT-PCR uses fluorescent probes to measure the PCR products formatted during each cycle of the PCR reaction. The fluorescence is recorded during the reaction and the cycle number of detection is proportional to the amount of target template in the samples. This contributes for the quantification of the viral nucleic acids in the samples (OIE, 2015). Variants of PCR are also available such as multiplex and nested RT-PCR. The multiplex PCR contains more than one primer set to amplify more than one fragment of a cDNA sequence, being valuable for influenza virus subtyping in

only one reaction (Kowalczy and Markowska-Daniel, 2009). Nested RT-PCR involves the use of two primer sets and two successive PCR reactions, and the first set of primers, used in an initial reaction, are designed to anneal to sequences upstream from the second set of primers. PCR products from the first PCR reaction are used as templates for the second reaction that uses the second set of primers, resulting in a second amplification step (Carr, 2010). The sensitivity and specificity of the assay is enhanced using nested RT-PCR, however, the risk for PCR contamination increases (Mahony, 2008). The multiplex and nested PCR techniques can be combined, improving the sensitivity of the reaction and capacity to subtype different influenza viruses in only one reaction (Choi et al., 2002).

Direct or indirect immunofluorescent antibody testing (DFA or IFA) are tests that use primary or secondary antibodies linked to a fluorophore. When the antibodies bind to a target molecule (antigen or antibody) the fluorophores are detected by fluorescent microscopy. These types of tests can only distinguish between influenza A and B, but does not distinguish influenza A subtypes (Chauhan et al.,2012). Similarly to the fluorescence antibody test, the immunohistochemistry (IHC) also uses antibodies for the detection of antigenic proteins, normally the nucleoprotein NP. But the test is applied to sections of formalin-fixed tissue which is advantageous since the tissues are fixed, not occurring loss of its integrity and allows the association with lung lesions (Janke, 2000; Schaefer et al., 2012).

The primary serological test for the detection and quantification of swine influenza antibodies is the hemagglutination inhibition (HI) test, which is subtype and strain specific. The basis of the HI test is the inhibition of hemagglutination with subtype-specific antibodies. The viral glycoprotein HA has the ability to agglutinate erythrocytes of various species, however in the presence of antibodies specific for the researched hemagglutinin, the HA-erythrocyte binding is inhibited (Beard, 1980). The HI assay is rapid and a relatively inexpensive procedure being less technical than molecular tests (Pedersen, 2014), however problems related to genetic changes and non-specific hemagglutination inhibitors may occur compromising the test result and reliability (Wood et al., 1994). The enzyme-linked immunosorbent assay (ELISA) identifies antibodies against the NP protein, and only indicates if the animals are infected with influenza type A, non-differentiating the SIV viral subtypes, being valuable to determine the immune status of the herd (Ciacci-Zanella et al., 2010). Serum neutralization (SN) assay measures neutralizing antibodies capable of preventing the influenza virus from infecting cells (Leuwerke et al., 2008)

3.7. Control and Prevention

Swine influenza has a negative impact on pig production, either by the direct losses due to mortality, or significant decrease in the weight gain of the affected animals, or even by increased spending on drugs to treat secondary infections. In addition, influenza is a zoonosis, posing risks to human health. Once the disease is established in a swine herd, it is difficult to eradicated it without full depopulation, thus biosecurity measures are the most important for the control of IAV and for reducing the negative economic impact on pig production (Schaefer et al., 2013). Besides biosecurity, the correct nutritional management, animal welfare and vaccination are also important. Cleaning, disinfection, proper ventilation of facilities, partial depopulation, separation of diseased animals from the healthy, segregation of freshly weaned pigs and all-in-all system, are extremely important to prevent the spread of influenza virus (Flores et al., 2007; FAO, 2010).

The constant replacement and supply of swine from several sources in a herd constitute a risk, as it keeps the disease circulating in the herd and contributes also to the emergence of a new virus by facilitating viral rearrangement in pigs and virus transmission between them (Schaefer et al., 2013). Therefore, a correct introduction of animals on farms by means of quarantine, control of origin and adoption of downtime are important measures (Dias et al., 2015). As virus transmission may occurs among different species, biosecurity measures in the herd include also limited contact of pigs with other species, especially birds and humans. Vaccination of employees of the pig production chain is recommended because of the possibility of human transmitting influenza virus to swine and vice versa (Nelson et al., 2012).

Worldwide, the most widely used method for control and prevention of swine influenza is vaccination. Currently, commercial intramuscular inactivated vaccines are the most used (Chen et al., 2012). Depending on the region, live-attenuated vaccines are also available, existing also autogenous inactivated vaccines (Vincent et al., 2008). There is no formal system for recommending SIV vaccine strains, and either updates or strain selection is complicated, since prevailing SIV strains vary widely among regions. Additionally, multiple SIV subtypes and lineages circulate concurrently within each continent, and new lineages have emerged during the last 10–15 years (Van Reeth and Ma, 2012).

Generally, inactivated vaccines are administered in two doses ranging from 2 to 4 weeks between them. For sow herds, gilts are vaccinated twice before the first coverage and the sows

are vaccinated 3-6 weeks before farrowing. Piglets are usually vaccinated after 12 to 16 weeks of age to avoid maternal antibody (MDA) interference, especially in sow herds with high antibody levels to SIV (Kitikoon et al., 2006; Van Reeth and Ma, 2012).

Inactivated vaccines usually provide humoral and cellular immunity but have limited ability to cross-protect against heterologous viruses (Platt et al., 2011). Therefore, good protection occurs when the HA of the vaccine strain is similar to the HA of circulating virus (Van Reeth et al., 2004; Vincent et al., 2008). The cross protection between heterologous subtypes is variable. Some studies have shown that antibodies produced from the contact with H1N1pdm09 virus, do not induce enough protection against human H1N1 and no protection against H3N2 subtype (Chen et al., 2012).

In addition to the low cross-protection of vaccines, experimental studies in animals immunized with inactivated, monovalent vaccine, and subsequently challenged with an antigenically different strain showed an exacerbated clinical disease condition, called Vaccine Associated Enhanced Respiratory Disease (VAERD) (Gauger et al., 2011), which can occur when vaccinated pigs are challenged with heterologous viruses (Rajão et al., 2014). Piglets vaccinated with a delta-1 cluster H1N2 and challenged with H1N1pdm09 virus have demonstrated enhanced clinical signs of the disease and higher score of lung lesions compared to non-vaccinated/challenged animals (Gauger et al., 2011). It is supposed that IgG antibodies induced by the vaccine strain bind the heterologous strain, but fail to neutralize it, not providing protection and promoting virus infectivity and fusion, leading to elevated replication (Khurana,2013; Sandbulte,2015)

Currently, in North America and Europe there are commercial vaccines available against H1N1, H1N2 and H3N2. In Europe, licensed vaccines contain the subtypes H1N1, H3N2 and H1N2 as their antigenic strains. In the United States, vaccines for SIV were introduced for the classical virus H1N1 in 1994, and it is estimated that 90% of the American herd is vaccinated. The major challenge for vaccines for swine influenza is that circulating SIV is under constant evolution. In the U.S. it is currently estimated that 7 antigenic clusters and subtypes co-circulate in the swine population as H3N2, αH1, βH1, γH1, δ-1H1, δ-2H1 and pH1N1 (Vincent et al., 2014). Because of the SIV genetic variability, commercial vaccines in US are mostly multivalent having in their composition different subtypes and clusters (Sandbulte, 2015), but monovalent vaccine against H1N1pdm09 is also available (Van Reeth

and Ma, 2012). The use of autogenous vaccines in US has been increasing, because of the strain diversity that circulate in the swine population, and also because of the difficulty for biological industries to alter vaccine composition as rapidly as the virus evolves (Vincent et al., 2008).

In Brazil, a monovalent and inactivated vaccine encoding the hemagglutinin protein of the 2009 pandemic (A/California/04/2009/(H1N1) was licensed on May 2014 (Brasil, 2014). Previous studies have shown that a diverse SIAV lineages (H1N1, H1N2, and H3N2) circulate in swine herds, however, studies characterizing the viruses circulating in Brazil are scarce. Since the only available vaccine is against H1N1pdm09 and studies have pointed limited vaccine cross-protection against heterologous viruses, swine herds in Brazil are frequently susceptible to disease, even vaccinated.

Since the genetic variability of circulating SIV is wide, different geographically and constantly evolving, vaccine production is a challenge for the swine industry. Surveillance studies are necessary to identify and characterize circulating viruses, especially the HA glycoprotein, which is the main target of neutralizing antibodies.

4. Material and Methods

4.1. RT-PCR Standardization

4.1.1. Control samples

The H1N1pdm09, H3N2 and H1Delta positive control samples used for the PCR standardization were previously characterized by RT-PCR and sequencing (Rajão et al., 2013a; Costa, et al., 2015). These strains were isolated by passages in Madin-Darby Canine Kidney (MDCK) cell culture. All three strains were isolated from lungs fragments of animals with respiratory clinical signs, from pig farms in Brazil (Table 1).

Table 1. H1N1pdm09, H1Delta and H3N2 strains used as control for RT-PCR standardization.

Strain	State origin	Species	Collection year	Reference
H1N1pdm09	Minas Gerais	Swine	2009	Rajão et al., 2013a
H1Delta	Santa Catarina	Swine	2013	Costa et al., 2015
H3N2	Paraná	Swine	2013	Costa et al., 2015

4.1.2. Clinical samples

A total of 92 field samples, 71 lung tissues and 21 nasal swabs, positive for influenza A matrix gene by RT-PCR (OIE, 2015), from seven Brazilian states (Santa Catarina (SC),Paraná (PR), Rio Grande do Sul (RS), São Paulo (SP), Minas Gerais (MG), Mato Grosso do Sul (MS) and Goiás (GO)), sampled in the years 2012-2013, 2014-2015 and 2017-2018 were used to validate the SIV subtyping Nested RT-PCR. Samples were collected in pig farms with respiratory clinical signs and received for routine diagnostic at the Laboratório de Pesquisa em Virologia Animal (LPVA), Department of Preventive Veterinary Medicine, School of Veterinary Medicine of the Universidade Federal de Minas Gerais (UFMG). Seventeen samples were received in the years 2012-2013, forty-nine in 2014-2015, and twenty-six in 2017-2018. It was not possible to obtain samples from 2016. All samples from 2014-2015 were kindly provided by MICROVET Laboratory, located in the city of Viçosa, state of Minas Gerais, Brazil.

4.1.3. RNA extraction

For RNA extraction, clinical samples were previously diluted in PBS supplemented with penicillin (200 IU/mL), streptomycin (200 ug/mL), and amphotericin B (2.5 ug/mL). Lung tissue fragments were macerated using mortar and pestle and were diluted ten-fold in PBS. Nasal swabs were diluted five-fold and were homogenized using a vortex. Then, all samples were centrifuged for 10 min at 4500g, at 4$_{o}$C. RNA was extracted from 300 µL of the supernatant using Trizol® reagent (Thermo Fisher, USA) according to the manufacturer's instructions.

4.1.4. Primer design

Sequences of the HA glycoprotein gene from Brazilian swine influenza viruses were obtained from Genbank and aligned with the ClustalW multiple sequence alignment (Thompson, Higgins & Gibson, 1994). The sequences were aligned separately by subtype of HA (H1 or H3) and conserved regions of H1 and H3 were used to design the external primers. From the selected regions, internal conserved regions from H1N1pdm09, H1Delta and H3N2 were chosen to design the primers for the Nested RT-PCR. Genbank accession number for all the Brazilian SIV published sequences, considered for primer design, are shown on Supplementary Table 1.

One set of primers were designed for the first RT-PCR reaction targeting the H1 (H1F and H1R) and another set of primers for H3 (FW1H3 and RV1H3). For the second reaction, three different sets of primers were designed, targeting specific internal and conserved regions of H1N1pdm (FW2H1PDM and RV2H1PDM), H1Delta (FW2DELTA and RV2DELTA) and H3N2 (FW1H3 and RV2H3).

The information about the primers used for the PCR reactions assays are shown on Table 2, as well as their positions, amplified base pair fragments and respective melting temperatures.

Table 2. Sequence, positions, amplified base pair fragments and melting temperature of primers for the Nested RT-PCR of H1N1pdm09 and H1Delta subtypes.

Reaction/ Subtype	Set of primers	Sequence (5'-3')	Reference strains	Positions	Product size (bp)	Tm (°C)
1stReaction/ H1	FW1H1	AGCAAAAGCAGGGGAAAAYA	JQ666848.1	781-798	616bp	50
	RV1H1	CCCCATAGYAYRAGGACTTC		534-553		
2stReaction/ H1N1pdm	FW2H1PDM	CCAATCATGACTCGAACAAC		391-410	159bp	52
	RV2H1PDM	ATAGCACGAGGACTTCTTTCT		529-549		
2stReaction/ H1Delta	FW2H1DELTA	GCGTTGCCGGATGGATCTTA	KF680296.1	233-252	303bp	55
	RV2H1DELTA	ACAGACCATTCTTCCCCGTCA		515-535		
1stReaction/ H3N2	FW1H3	ATATGTTCAAACGTCAGGGAGA	KM507511.1	651-672	544bp	52
	RV1H3	GTCGATTGCTGCTTGAGTGC		1175-1194		
2stReaction/ H3N2	FW1H3	ATATGTTCAAACGTCAGGGAGA		651-672	279bp	52
	RV2H3	TTCCCATTTGGAGTGACGCA		910-929		

4.1.5. One Step and Nested RT-PCR for SIV subtyping

For the first reaction, the viral genome was reverse transcribed and amplified using Superscript One-Step RT-PCR System with Platinum Taq DNA polymerase (Life technologies, Carlsbad, CA, USA). All samples were submitted to a standardized 25µL PCR reaction containing 0.4µM of each primer, 200µM of each dNTP, 2.5µL of the extracted RNA, and concentration variations of $MgSO_4$ (1.5mM and 2mM) and different volumes of Taq (0.6 µL and 1µL) were tested for standardization.

One microliter of the first RT-PCR reaction was used as template for the nested RT-PCR internal reaction. The second reaction was carried by 24 µL of master mix containing

0.5µM of each primer, 1.5mM $MgCl_2$, 200µM of each dNTP and 3U of Taq polymerase (Phoneutria Biotecnologia, Brazil).

The cycling conditions were performed according to the manufacture's kits and annealing temperatures (Ta) for the realization of One Step RT-PCR and Nested RT-PCR reactions were calculated from Tm (melting temperature) of the chosen primers. Four different temperatures were selected to evaluate the ideal Ta for each primer pair: 58°C, 55°C, 52°C and 50°C. The PCR amplicons were identified by electrophoresis in a 1% agarose gel and stained by ethidium bromide.

4.1.6. Sensitivity of the assay

The method of limiting dilution was used to evaluate the analytic sensitivity of the Nested and One Step RT-PCRs. The primers sets were tested for the detection of the three reference swine influenza viruses. H1N1pdm09 and H3N2 isolates, with titers of $4x10^5$ plaque forming units (PFU)/ml, were 10-fold serial diluted (10^{-1} up to 10^{-14}) and the RNA was extracted from 300µL of each dilution.

It was not possible to titrate the H1Delta, once the material was already extracted. From the extracted RNA, a 10-fold serial dilution was performed.

4.1.7. Specificity of the assay

In order to test the specificity of the assay, all sets of primers from the second amplification of the Nested RT-PCR reaction were tested for each reference sample. In addition, the specificity of the test was also evaluated by sequencing the PCR products from the field samples.

4.1.8. RT-PCR to differentiate the N gene in H1Delta positive samples

To differentiate positive samples of H1Delta into H1N1Delta and H1N2Delta, specific primers as well as cycling temperatures for the detection of N1 and N2 were used according to Choi et al (2002).

4.2. Serological analysis

For serological analyzes, samples received at the LPVA for routine diagnostic, from non-vaccinated farms located in different Brazilian states in 2017 and 2018 (Table 3) were tested. A total of 940 samples were analyzed in this period, 263 samples from 2017 and 677 from 2018 (Table 3).

Table 3. Location, number of farms and number of samples analyzed for the detection of antibodies against SIV in the years 2017 and 2018.

Year 2017		
State	**Farms**	**Samples**
MG	6	263
Year 2018		
State	**Farms**	**Samples**
MG	4	170
SC	2	441
GO	1	46
MT	1	20
Total	**14**	**940**

4.2.1. Hemagglutination (HA)

Reference samples of H1N1pdm09 and H3N2 (Table 1) were submitted to the hemagglutination test for the detection of viral titer in hemagglutinating units (UHA) (WHO, 2002). The viral titer was determined by the reciprocal of the last dilution where complete agglutination of the red blood cells occurred. Viruses were used for retro-titration in the hemagglutination inhibition (HI) test.

4.2.2. Hemagglutination inhibition (HI)

All serum samples were tested by HI, as previously described by WHO (2011). Briefly, serum samples were inactivated at 56°C for 30 minutes and treated with turkey red blood cells for the removal of non-specific hemagglutination inhibitors and natural serum agglutinins. Then, the HI test was performed to detect antibodies against viral suspension of H1N1pdm09 and H3N2, previously described on Table 1. Negative control containing PBS and red cell

suspension and also serum control to detect hemagglutination, containing only serum and red cell suspension, were used.

Sera were initially diluted to 1:20 and 2-fold serial diluted in PBS until the final dilution 1:640 using 96 well "V" bottom plates. Antibody titers from each sample were determined as the reciprocal of the highest dilution in which no hemagglutination was observed. Titers greater than or equal to 40 were considered positive.

4.3. Sequencing analyzes

The RT-PCR amplicons from subtyped field samples were purified using the DNA Clean and Concentrator kit (ZYMO Research, USA). Subsequently, the concentration of the purified amplicons were dosed by NanoDrop spectrophotometer (Thermo Fisher, USA) selecting those with more than 40ng for sequencing, according to the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher, USA). Sequencing reactions were performed by capillary electrophoresis in an ABI 3730 equipment (Applied Biosystems, USA). The nucleotide sequence data were analyzed and assembled using SegMan Software 7.1.0 (DNAStar Inc.). The consensus sequences were compared with those reported in the GenBank, selecting those with an identity percentage higher than 95%, using BLAST (Basic Local Aligment Search Tool) from the NCBI (National Center for Biotechnology).

4.4. Phylogenetic analyzes and p-distance of sequences from each subtype

Phylogenetic analyzes were performed with the MEGA 7.0.26 software (Kumar et al., 2016; Tamura, 1992). Using the MUSCLE alignment method (Tamura, 1992), the consensus sequences were aligned with reference strains and sequences of swine and human influenza viruses available on Genbank database. Phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992) and 1,000 bootstrap replicates of the partial sequence of the three subtypes positive samples.

Additionally, the sequences were compared within each subtype to identify the similarity between them. Sequences for each subtype were aligned using ClustalW multiple sequence alignment (Thompson et al. 1994) on Bioedit version 7.2.5 software (Hall, 1999). The pairwise distance were estimated by p-distance method under 1,000 bootstrap replications using Mega 7.0.26. software (Kumar et al., 2016).

4.5. Amino acid analysis

Sequences from the first reaction of H1N1pdm09 positive samples in the Nested RT-PCR were translated into amino acids and aligned, through the Bioedit version 7.2.5 software (Hall, 1999), with Brazilian H1N1pdm09 sequences detected in swine from 2009-2010 and HA sequences from three H1N1pdm09 vaccine samples used in vaccines against this subtype in human [(A/Michigan/45/2015 (H1N1)pdm09 and A/California/07/2009(H1N1)] and swine (A/California/04/2009) in Brazil. ClustalW multiple sequence alignment (Thompson et al., 1994) was used to identify amino acid variations /substitutions among all these partial HA sequences.

5. Results

5.1. One Step and Nested RT-PCR for SIV subtyping

The concentration and volume of $MgCl_2$ and Taq selected for the One Step RT-PCR reaction were 2mM and 0.6µL, respectively, which minimized the occurrence of nonspecific bands.

Selected annealing temperatures that minimized the occurrence of non-specific bands were 50°C or 52°C (for H1 and H3, respectively) for One Step RT-PCR and 52°C, 52°C and 55°C (for H1N1pdm09, H3N2 and H1Delta respectively) for the Nested RT-PCR.

Therefore, cycling conditions for the first RT-PCR reaction was an initial reverse transcription at 48°C or 50°C (for H1 and H3 respectively) for 30 minutes, followed by a denaturation step at 95°C for 2 minutes and then 40 cycles of denaturation at 95°C for 30s and annealing at 50°C or 52°C (for H1 and H3, respectively) for 30s, extension at 64°C for 1 minute, followed by a final extension step at 68°C for 5 minutes.

Second round of PCR started with a denaturation temperature of 95°C for 5 minutes followed by 40 cycles of a denaturation step at 95°C for 30 s, annealing at 52°C, 52°C and 55°C (for H1N1pdm09, H3N2 and H1Delta respectively) for 30s, extension at 68°C for 1 minute and a final extension step at 68°C for 5 minutes.

5.2. Sensitivity and specificity of the nested RT-PCR

To access the sensitivity of nested RT-PCR, a 15-fold dilution for H1N1pdm09 and H3N2 and a 10-fold dilution for H1Delta were performed. The sensitivity results of the assay are shown in Figure 4.

The limit of detection by nested RT-PCR for H1N1pdm09 was 10^{-11}, which was 10^{8} higher comparing to the one-step RT-PCR, which uses the first set of primers. For H3N2, the limit of detection was 10^{-9}, differently from the first reaction that was only 10^{-5}. The detection limit for H1Delta was lower, corresponding to 10^{-4} near the limit of the first reaction that was 10^{-3}.

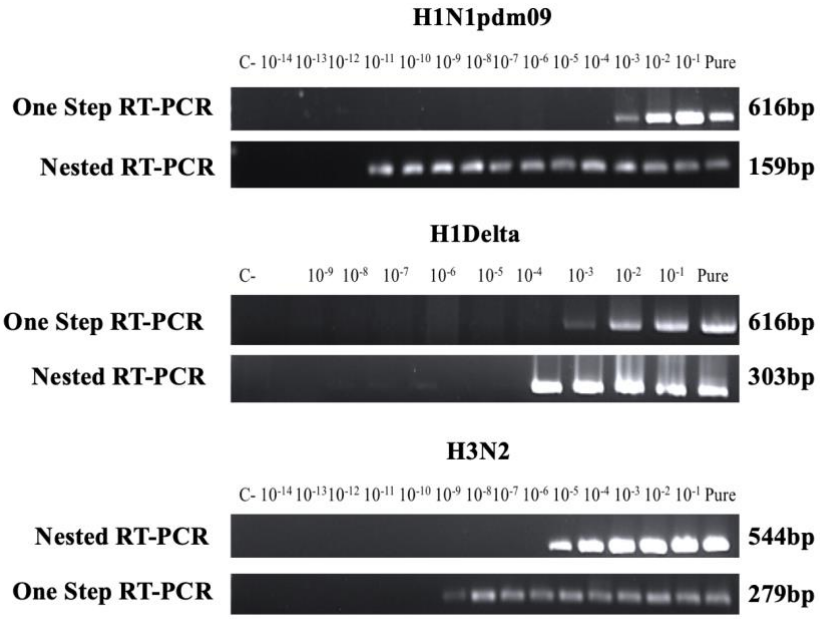

Figure 4. Agarose gel electrophoresis showing the first and nested RT-PCR reaction for the detection of H1N1pdm09, H1Delta and H3N2. The H1N1pdm09 and H3N2 control samples were submitted to a 15-fold dilution and H1Delta a 10-fold dilution. Amplified products for the first reaction of H1 and H3N2 correspond to 616bp and 544bp respectively, and for the Nested RT-PCR of H1N1pdm09, H1Delta and H3N2 amplified products have a band of 159bp, 303bp and 279bp, respectively.

To access the specificity of the assay, the three SIV reference samples (H1N1pdm09, H1Delta and H3N2) were tested with all three sets of primers used in the nested RT-PCR, separately. All the three subtypes yielded the expected base pair fragments, which are 159bp, 303bp, 279bp for H1N1pdm09, H1Delta and H3N2, respectively. It may be noted that when

the H1N1pdm09 sample was tested with the H1Delta primers, a band near 600bp was detected, evidencing the amplification of the fragment related to the first RT-PCR reaction (616bp for H1), but there was no amplification of the 303bp band that is specific for H1Delta (Figure 5b).

When cross-tested against non-specific primers for each viral subtype, none of the samples yielded any amplification product (Figure 5).

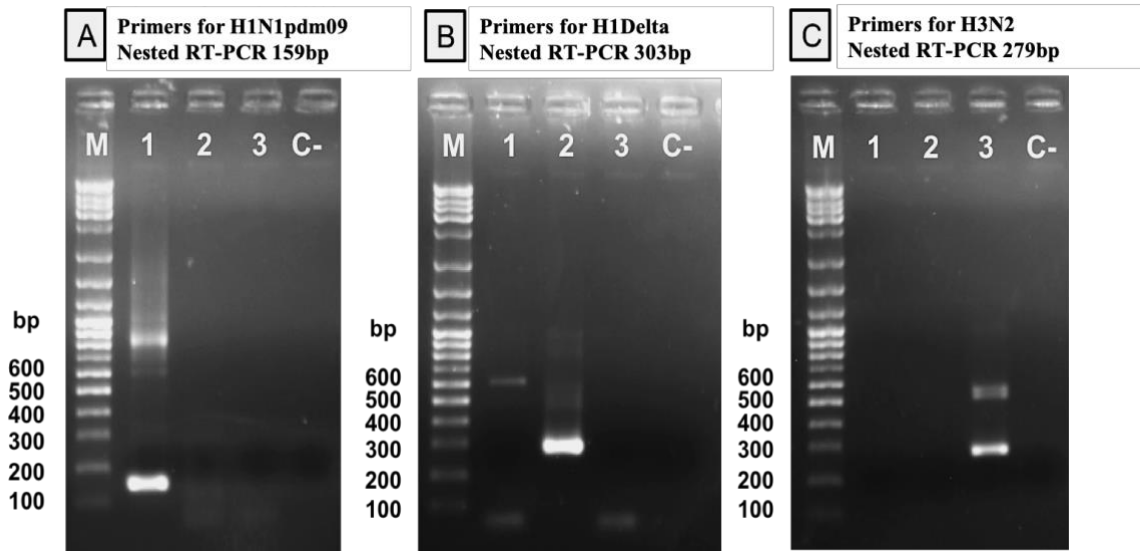

Figure 5. Specificity of the Nested RT-PCR assay for the three subtypes of SIV. A, B and C correspond to reactions tested with primers for H1N1pdm09, H1Delta and H3N2, respectively. Lane M contained the molecular size standards. Lane 1, 2 and 3 correspond to reference samples for H1N1pdm09, H1Delta and H3N2. Lane C- is the negative control of the reactions. Amplified products for H1N1pdm09, H1Delta and H3N2 are related to bands of 159bp, 303bp and 279bp, respectively.

In addition, to confirm the specific amplification of the nested PCR performed with the primers designed in this study, the amplicons were sequenced and analyzed by molecular phylogeny (Figure 9). The partial genome sequence of field samples indicated the specific subtype in accordance with the nested RT-PCR results.

5.3. Nasal swab and lung tissue field sampling

From the 92 samples positive for Influenza A matrix gene, 71 (71/92-77%) were subtyped by the nested RT-PCR developed in this study. Fourteen samples (14/71-20%) were from 2012-2013, 35 (35/71-49%) from 2014-2015 and 22 samples (22/71-31%%) from 2017-2018. Information about the subtyped and non-subtyped samples are present on Table 4.

Relation about subtyped and non-subtyped samples per state is listed on the Supplementary Table 2.

Table 4. Absolut ratio of subtyped and non-subtyped samples from the previously IAV positive samples.

	2012/2013	2014/2015	2017/2018	Total
Subtyped samples	14	35	22	71
Non subtyped samples	3	14	4	21
Total of analyzed samples	17	49	26	92

The percentage of subtypes detected by year are shown in Figure 6. From 2012-2013, 64% (9/14) of the subtyped samples were positive for H1N1pdm09 and 21% (3/14) were positive for H1Delta. Co-infection between H1N1pdm09 and H3N2 was observed in 14% (2/14) of the samples. However, in this period it was not observed H3N2 single infection, neither co-infection between the three subtypes (Figure 6). Relation of detected subtypes per state and years are shown on Supplementary Table 3.

In 2014-2015, the results of subtyping showed that 77% (27/35) of the samples were positive for H1N1pdm09, and only 3% (1/35) and 11% (4/35) were positive for H3N2 and H1Delta, respectively. In these years, co-infection was observed only between H1N1pdm09 and H1Delta, corresponding to 9% (3/35) of the subtyped samples (Figure 6).

Analyzing SIV subtypes occurrence in the years 2017-2018, the percentage of positive samples for H1N1pdm09 was 23% (5/22), which is lower when compared to the other years. However, an increase of H3N2 samples to 23% (5/22) was observed. Positive samples for H1Delta had a higher percentage compared to H1N1pdm09 and H3N2 corresponding to 27% (6/22). Co-infection between the three subtypes was detected in 14% (3/22) of the samples. In addition, when analyzing the co-infection between the pairs, H1N1pdm09+H3N2, H1N1pdm09+H1Delta and H3N2+H1Delta it was observed an occurrence of 5% (1/22) for each combination (Figure 6).

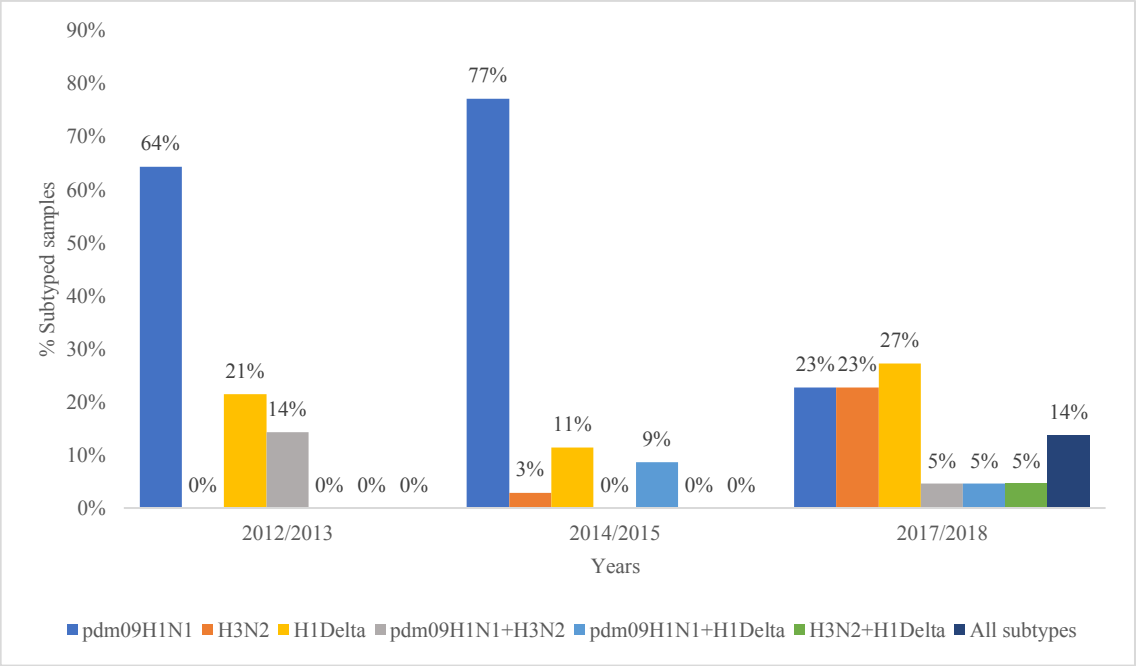

Figure 6. Percentage of the subtyped samples in the years 2012-2013, 2014-2015 and 2017-2018.

The relative occurrence of H1N1pdm09, H3N2 and H1Delta by years, considering the total of performed subtyping analyzes and not considering co-infection among subtypes, is demonstrated in Figure 7. In 2012-2013 the most detected subtype was H1N1pdm09 (69%-11/16), followed by H1Delta (19%-3/16) and H3N2 (13%-2/16). In 2014-2015, H1N1pdm09 remained the subtype with the highest percentage of detection (79%-30/38), followed by H1Delta (18%-7/38) and H3N2 (3%-1/38). However, in 2017-2018, the subtypes occurrence was equivalent, H1N1pdm09 and H3N2 were detected in 32% (10/31) of the samples and H1Delta in 35% (11/31).

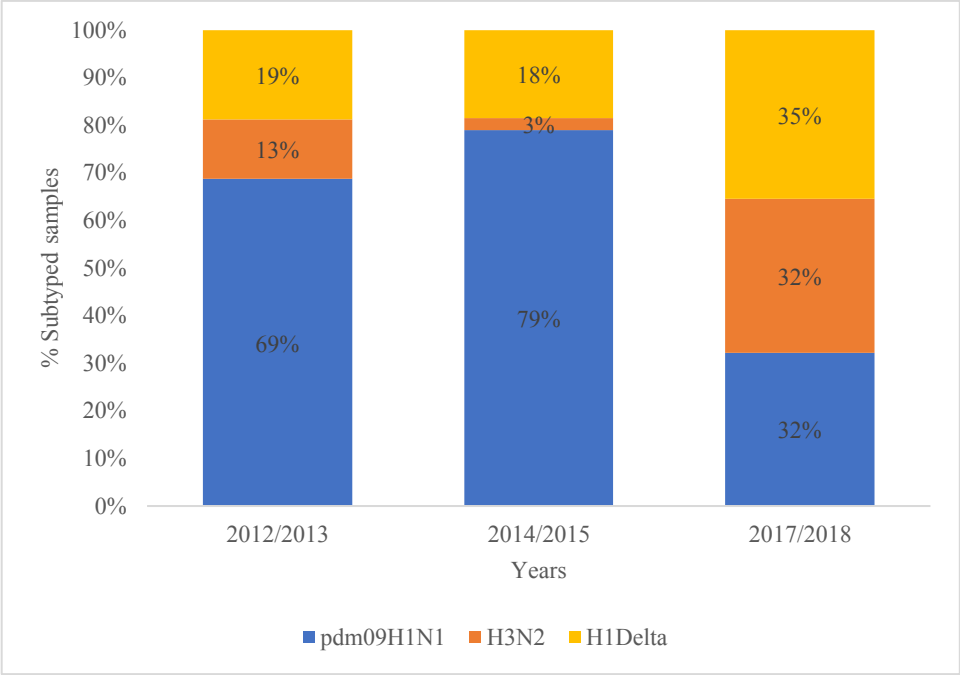

Figure 7. Percentage of positive samples for H1N1pdm09, H3N2 and H1Delta in relation to the total of subtyping analyzes performed, not considering co-infection between subtypes.

The 21 positive samples for H1Delta by the subtyping Nested RT-PCR were submitted to a RT-PCR to differentiate the N gene into N1 or N2, according to Choi et al. (2002) (Table 5). After confirming the N subtype, positive samples for H1Delta were classified into H1N1Delta or H1N2Delta.

Table 5. Absolut and percentage of H1Delta samples subtyped for the N gene.

	N1	**N2**	**Non-subtyped**
2012/2013	1/3 (33%)	2/3 (67%)	0/3 (0%)
2014/2015	3/7 (43%)	4/7 (57%)	0 (0%)
2017/2018	1/11 (9%)	1/11 (9%)	9/11 (82%)
Total	5/21 (24%)	7/21 (33%)	9/21(43%)

5.4. Serological Analysis

From the 940 serum samples analyzed by HI test, 707 (75%) were positive for IAV. Among the 233 negative samples, 37 (14%) and 196 (29%) were from 2017 and 2018, respectively (Figure 8). In 2017, the occurrence of single infection of H1N1pdm09 and H3N2 were 11% (28/263) and 33% (86/263), respectively. The highest occurrence was observed in H1N1pdm09+H3N2 co-infection which was 43% (112/263). In 2018, samples only positive for

H3N2 (40%-270/677) were more prevalent than H1N1pdm09 (3%-23/677). A high occurrence of H1N1pdm09+H3N2 (28%-188/677) (Figure 8) was observed.

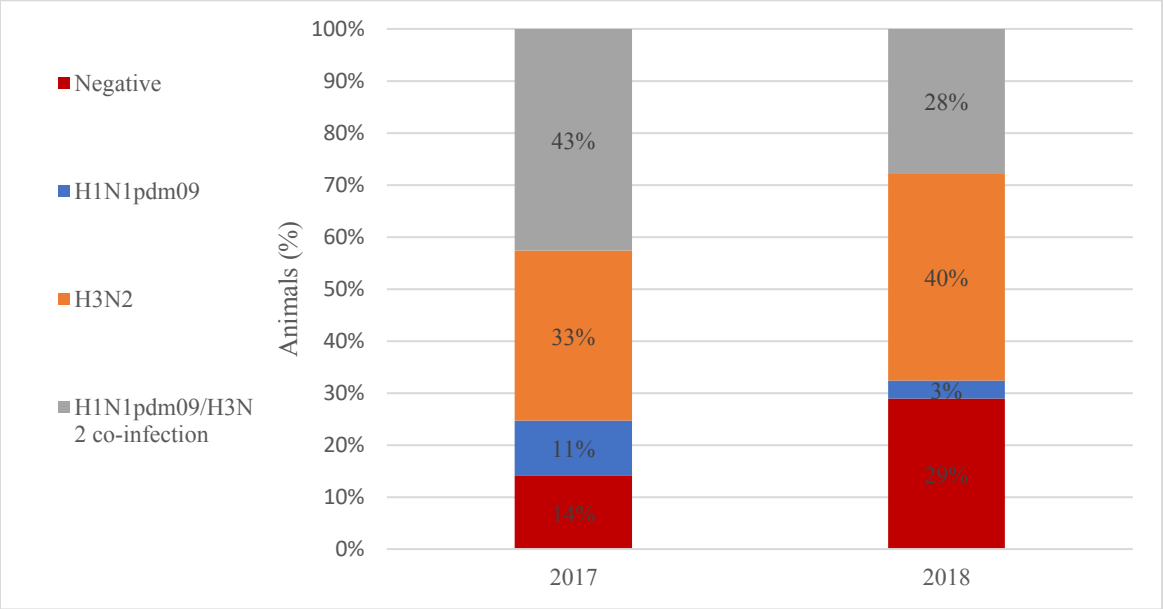

Figure 8. Percentage ratio of samples tested for antibodies against H1N1pdm09, H3N2 and H1N1pdm09+H3N2 in the years 2017-2018.

5.5. Sequencing, phylogenetic analysis and p-distance between sequences from the same subtype.

The amplicons from the first reaction of the 23 positive samples by RT-PCR for H1N1pdm09, were sequenced. Because of the small size of nested RT-PCR products, it was not possible to sequence the PCR products from the second reaction. PCR products from the second reaction of H1Delta and H3N2 were sequenced totalizing thirteen and nine samples for each subtype, respectively. Sequences from the first reaction of H1N1pdm09 had a size ranging from 320-544bp, and from the second reaction of H1Delta and H3N2, a 150-303bp and 190-279bp size ranging, respectively. The molecular phylogenetic analysis for H1N1pdm09, H1Delta and H3N2 samples is shown on Figure 9. The relation of sequenced samples number per state and subtype, and per year and subtype are listed on the Supplementary Table 4 and 5, respectively.

All the H1N1pdm09 sequenced samples (labeled in blue) grouped in the same cluster. Same observation was noticed when analyzing the H1Delta sequences (labeled in yellow) and H3N2 samples sequences (labeled in orange) (Figure 9).

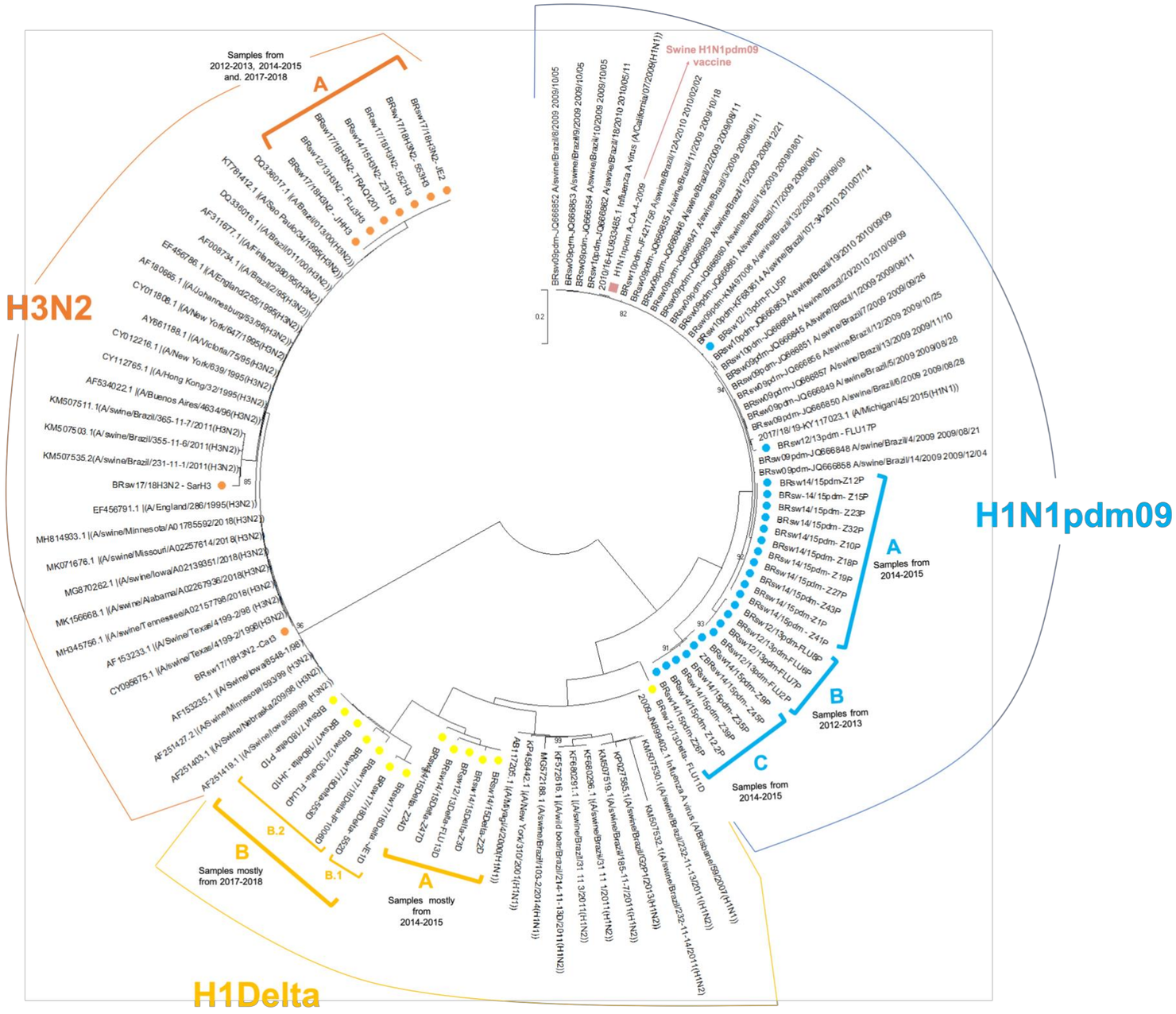

Figure 9. Molecular phylogenetic tree of the partial sequences of H1N1pdm09 (labeled in blue), H1Delta (yellow) and H3N2 (orange) positive samples from this study. Non-labeled sequences refers to published Genbank sequences of the three subtypes. Sequences of H1N1pdm09 were distributed in 3 groups (A,B,C). H1Delta in 2 groups (A and B). And H3N2 in one group (A). Molecular phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992) and 1,000 bootstrap replicates of the partial sequence of the three subtypes positive samples.

The similarity between the samples from the same subtype were estimated by p-distance method (Kumar et al., 2016). It was observed that H1N1pdm09-like samples presented 88-100% of similarity, while H1Delta and H3N2 indicated a similarity of 75-100% and 91-100%, respectively (Supplementary Table 6, 7 and 8).

H1N1pdm09 sequences were grouped into 3 different groups (A, B and C), and were related to different years. Two sequences (Flu17P and Flu5P), from 2012/2013, have aligned separately (Figure 9). Analyzing the p-distance between the samples (Supplementary Table 5), in group B most of the samples from 2012/2013 have grouped with 100% of similarity. Similarity of this group to the sequences that lined up separately, was 96% -97%. These two samples were more similar to the 2009- 2010 samples and also to the swine vaccine strain (98-99% similarity). Samples of H1N1pdm09 from 2014/2015 were divided into 2 groups (A and C) (Figure 9). Within each group, samples had 99-100% of similarity between them and group B and C were 12% different. Group A was the one that most resembled the 2009 samples and the vaccine (98-99% similarity) and group C had a lower similarity when compared to these samples (89-90%). When comparing the sample sequences from 2012-2013 (Group B) with group A and C (2014/2015), similarity of 96% and 86-88% was found, respectively.

From the cluster formed with the H1Delta samples, two distinct groups (A and B) and a separated sample (Flu11D) were observed (Figure 9). Analyzing the p-distance within this cluster (Supplementary Table 6) only the 2012/2013 sample lined up separately and had 17-25% dissimilarity with all H1Delta samples. Samples of group A were mostly from 2014/2015 and had 98.5-100% similarity to each other. Group B was composed mostly of samples from 2017/2018 and the similarity between them ranged from 86-100%, since this group have subdivided into subgroup B.1, composed of sequences with 100% similarity between them, and B.2 with 96-100%. Group A and B of H1Delta were 85-94% similar to each other. Group A had 93-97% similarity with samples of human H1N1 and 89-100% with H1N1 and H1N2 isolated from swine and wild boars. Group B had 90-97% similarity between the samples of human seasonal H1N1 and 86-97% between swine and wild boar H1N2.

When analyzing the distribution of H3N2, all samples grouped into a single group (Group A) except the Cat2 and SarH3 sample (Figure 9). Sequences of group A had 99.5-100% similarity among themselves and with Cat2 partial sequence sample (Supplementary Table 7), and the majority of these samples were from 2017/2018. Comparing Group A and Cat2 sample

with Genebank sequences it was observed a high similarity (96-100%) with swine H3N2 North American sequences and human H3N2 from different countries of the world. Low similarity was observed with swine Brazilian H3N2 and SarH3 sequences. The SarH3 differed in 7-9% of all H3N2 sequences, except with swine Brazilian H3N2 which had 96% similarity.

5.6. Amino acid variation analysis of H1N1pdm09 partial sequences

The amino acid alignment between partial sequences of H1N1pdm09 of field samples from this study (years 2012-2013 and 2014-2015), H1N1pdm09 of SIAV from Brazil in 2009-2010 (available on Genbank database) and swine and human vaccine strains used in Brazil have shown some residual variations (Figure 10). The differences between the amino acid residues are indicated in the red marked regions at Figure 10. Since the sequences of samples were partial, only a few residues of the antigenic sites Cb (74-79), Sa (128-129) and Ca2 (140-145) could be noted. It is important to observe that in six sequences from 2013-2014 there were amino acid variations at residues 78 and 144, which belongs to the Cb and Ca2 antigenic sites. In other four sequences, from 2011-2012, punctual variation in residue 129, which belongs to the antigenic site Sa, were present. Some binding site residues (99, 133-139) and conserved glycosilation sites (19-22; 34-36; 95-97), as described in the literature (Sriwilaijaroen and Suzuki, 2012), could be detected in our partial sequences, and it was not observed any alteration in these regions.

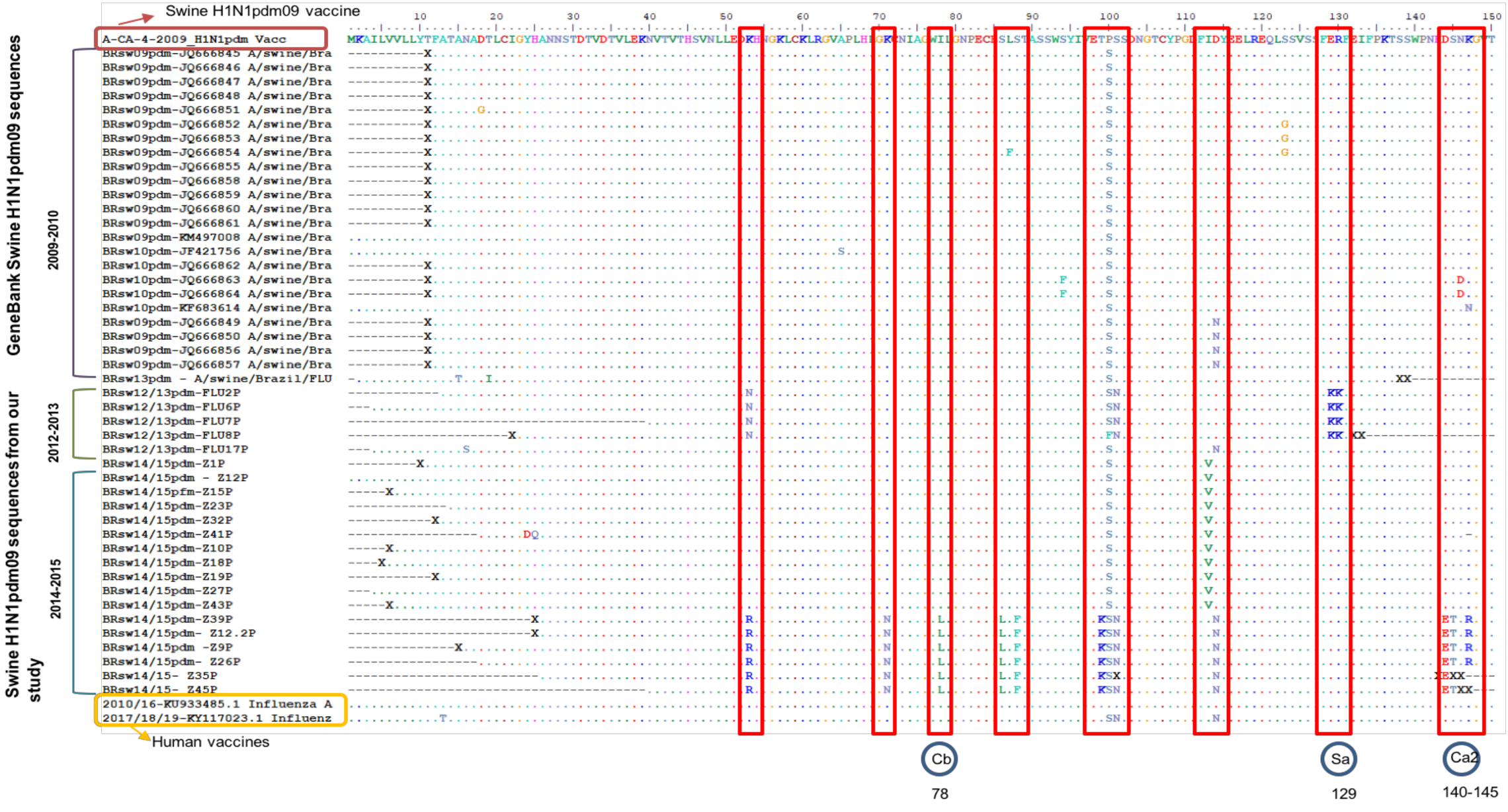

Figure 10. Analysis of amino acid residues from partial sequences of HA protein using the alignment between the swine vaccine strains licensed in Brazil (first sequence), sequences of H1N1pdm09 previously deposited in Genbank 2009-2010, H1N1pdm09 sequences from our study (2012 -2015) and human vaccine strains sequences (last two alignment sequences). Antigenic sites Cb (residue 78), Sa (129), Ca2 (140-145) are identified in the lower part of the figure. Red stripes highlight changes in amino acid residues.

6. Discussion

Brazil is the fourth largest producer of swine in the world, and responsible for almost 9% of the pork volume exported worldwide (ABPA, 2017). Despite the highly favorable data on pig production, infectious diseases are currently an important issue in swine farming, specifically respiratory infections, as SIV (Piffer and Brito, 1993; Maes et al., 2008). A rapid and accurate diagnosis is essential for assertive actions to minimize the damage caused by infectious diseases in the herds.

Although the importance of a rapid diagnosis, in Brazil there is no diagnostic test commercially available to differentiate swine influenza subtypes in herds. Generally, studies for the detection and differentiation of SIV subtypes use the multiplex PCR technique that identify only H1 or H3, but they cannot distinguish H1 between H1Delta or H1N1pdm09 (Gava et.al, 2011). To characterize and differentiate circulating SIV, studies conducted in Brazil firstly identify the positive samples by PCR targeting the matrix gene, then inoculate the positive samples in embryonated eggs or MDCK cells to further submit for genome sequencing by Next Generation Sequencing (NGS), obtaining complete viral characterization (Schaefer et al., 2015; Zanella,2015). Although the method of detection and characterization of SIV used in these studies were very efficient to obtain complete sequences of circulating viral strains, the diagnosis of viral subtypes of SIV was not feasible, since it is a laborious, expensive and time-consuming process to be used in routine. Rapid, sensitive and specific diagnostic tests for the detection of different SIV subtypes are necessary and essential for monitoring circulating viruses in Brazil.

The Nested RT-PCR test developed in the present study showed satisfactory results, since the three subtypes analyzed (H1N1pdm09, H1Delta and H3N2) amplified both reference and field samples, without nonspecific amplifications. It is important to emphasize that the test was standardized using primers delineated from Brazilian swine influenza sequences, increasing the specificity of the test and its importance of use to subtype field samples monitoring SIV in Brazil.

Regarding the sensitivity of the proposed Nested RT-PCR assay, it was possible to detect up to 11 ten-fold dilutions of reference strain H1N1pdm09, and up to nine ten-fold dilutions of H3N2, that had initial viral titer of $4x10^5$ PFU/ml. The detection of the two subtypes

within these limits was interesting because it shows that the Nested RT-PCR method can identify low concentrations of genetic material present in clinical samples. This is very important when evaluating field samples, since there may be variation of viral load according to the moment of sample collection, quantity and quality of the clinical sample.

The field samples used in this study were received in the LPVA-UFMG for routine diagnosis, thus, it was not possible to control some details that may interfere the RT-PCR, such as collection and storage of samples prior to arrival at the laboratory. However, it was possible to subtype 71 out of 92 samples positive for IAV. It is known that M protein is the most abundant in the IAV virion (Lamb et.al, 2001) and due to this it was possible to detect only the M gene in the 21 non-subtyped samples, possibly due to low viral load or low concentration of RNA extracted from the field samples. In addition, stability studies of IAV viral RNA showed that the M gene, due to structural characteristics, is more resistant than the HA gene under different storage conditions (Relova et. al, 2018). The high sensitivity of the assay demonstrated herein certainly improved the success rate of subtyping. The sensitivity of Nested RT-PCR to H1Delta was lower when compared to the sensitivity of the other two subtypes. However, it is important to highlight that the H1Delta sensitivity test was performed using extracted RNA diluted ten-fold, while the other virus strains were previously diluted and then submitted to RNA extraction, which may influence the amount of virus genome present in the analyzed dilution. In addition, when comparing the limit of detection between one-step RT-PCR and the nested RT-PCR to H1Delta, the difference was not so evident, although the second reaction increased the detection of viral RNA in one ten-fold dilution.

The Nested RT-PCR assay is a very sensitive method when compared to other diagnostic techniques and it is considered a rapid test. However, it is associated with a high risk of cross-contamination. In our study some precautions were taken to prevent contamination, since reagent preparation, sample processing, Nested RT-PCR and run of generated PCR products were performed in different rooms. The use of RNA and DNAse free filter tips, as well as pipette cleaning between each application of samples were adopted in the experiment, minimizing the chances of contamination.

The specificity of the standardized nested RT-PCR assays was also demonstrated as amplified fragments, specific for the reference samples, were only detected when tested with the specific primers for each subtype. The high specificity could also be noted by RT-PCR

amplicons sequencing, since specific sequences were detected for H1N1pdm09, H1Delta and H3N2, demonstrated by distinct clusters formed in phylogenetic analysis with in each reference strain (Figure 9).

The distinction between H1N1pdm09 and H1Delta using nested RT-PCR demonstrated to be an interesting tool to SIV diagnosis in Brazil, since the majority of previous studies detected only the subtype H1 or H3, not distinguishing H1 from H1N1pdm09 or H1Delta.

Another favorable aspect of the study was the primers, as they were designed based on SIV sequences available on GenBank database of Brazilian strains, which increases the ability of the test to detect subtypes circulating in Brazil.

In addition to the high specificity and sensitive, the nested RT-PCR developed ended as a rapid diagnostic method, and in less than 24 hours it is possible to obtain subtyping results from clinical samples, helping field veterinarians to quickly establish measures to prevent and control the disease. The nested RT-PCR for SIV subtyping is a faster and cheaper technique when comparing to genomic sequencing and allows to monitor the prevalence of different circulating SIV subtypes in swine herds. In this study, using the standardized technique, it was determined the occurrence of the SIV subtypes in field samples from 2012-2013, 2014-2015, 2017-2018. Between 2012-2013, was observed a higher detection of H1N1pdm09, followed by H1Delta and H3N2 + H1N1pdm09 (Figure 7). A seroprevalence study performed in the state of Minas Gerais in 2012, showed that 26.2% of the tested animals had antibodies against H1N1pdm09 and only 1.57% for H3N2. Additionally, it indicated that 96.6% of the herds were positive for H1N1pdm09 and 13.2% for H3N2 (Dias et al., 2015). Another study conducted in 2011 in the states RS, PR, SC, SP, MG and MS also showed that H1N1pdm09 subtype was more prevalent in the herds and animals analyzed, followed by H1N2 and H3N2 (Ciacci-Zanella et al., 2015). High prevalence of co-infection among different subtypes, with greater involvement of H1N1pdm09, was also observed (Ciacci-Zanella et al., 2015).

As observed in the years 2012-2013, samples collected in 2013-2014 showed also a higher occurrence of H1N1pdm09, followed by H1Delta and a low detection of H1Delta + H1N1pdm co-infection. The subtype H3N2 was the least detected subtype in these years but, unlike the earlier period, H3N2 was not detected in co-infections. A serological study performed by our group in 2014-2015, in unvaccinated farms from several Brazilian states, showed that 96% of the herds were positive for H1N1pdm09 (Fraiha et al., 2017). This data

corroborates the data found by this RT-PCR detection study, since in this period H1N1pdm09 was the most frequently detected subtype. In the serological study previously performed (Fraiha et al., 2017), 68% of the farms were infected with H3N2, and from these, and 36% presented co-infected animals with H1N1pdm09. Another study which have evaluated the detection of SIV subtypes in Brazilian swine samples, previously positive for IAV in the years 2010-2016, showed that the most prevalent subtypes detected were H1N1 (64.9%), H1N2 (29.8% %) and H3N2 (5.3%) (Haach et al., 2018).

The higher occurrence of H1N1pdm09 in the periods 2012-2013 and 2014-2015, demonstrated in the present study, indicates that in these periods, H1N1pdm09 was endemic with a high prevalence in Brazilian swine population. Co-infections between the subtypes were also observed and call attention to the higher probability of occurrence of rearrangements between different subtypes, since these events may have been favored by the introduction of H1N1pdm09 in the country in 2009 (Nelson et al., 2015). In addition, the high detection of H1N2 in these studies reinforces that this human origin subtype circulates in the Brazilian swine herds (Nelson et al., 2015; Schaefer et al., 2015b) and seems to have settled in this population, since is rare its occurrence in the human population (Resende et al., 2017). Although the detection of H3N2 by RT-PCR was low in 2014-2015, it increased when comparing to the previous years, which was also demonstrated by previous serological studies (Fraiha et al., 2017). These findings suggest a trend of virus circulation among the swine population over the years.

In 2017 and 2018, SIV RT-PCR subtyping data showed a significant increase in the detection of H3N2 and a decrease in H1N1pdm09, comparing to previous years. Additionally, serological data, from unvaccinated farms, indicated that the occurrence of H1N1pdm09 decreased comparing to the data from previous years, and there was a significant increase in H3N2 as well as H1N1pdm09 + H3N2 co-infection, already indicated in 2014-2015 studies (Fraiha et al., 2017). The serological results corroborate the RT-PCR findings, showing a significant decrease in H1N1pdm09 and an increase of H3N2 detection in clinical samples. These results are interesting since our study have demonstrated for the first time a higher occurrence of H3N2 in recent years. Compared to other years, RT-PCR test showed that co-infection detected in 2017-2018 was more frequent, reinforcing the fact that since the introduction of H1N1pdm09 the possibility of reassortments increased in swine herds, both in Brazil and in other countries (Nelson et al., 2015).

Analyzing the serological data from 2017-2018, the increasing occurrence of H3N2 may also be related to the occurrence of this IAV subtype in human population. In 2017, a greater and significant occurrence of H3N2 was observed in Brazilian human population, while the occurrence of H1N1pdm09 was not very expressive (WHO, 2018) (Figure 2). Low occurrence of H1N1pdm09 may be related to the introduction of a new H1N1pdm09 vaccine strain into the human vaccine formulation. It is important to note that in 2017, in swine population, H3N2 was also the most prevalent subtype, evidencing a parallel occurrence of H1N1pdm09 and H3N2 in both species.

In 2018, there was an increase of H3N2 in swine population, which may be related to a greater contact of humans with these animals, since in the previous year this subtype was the most prevalent in human population (WHO, 2018; Figure 2). Events of influenza transmission from humans to pigs have been shown to be more frequent than the reverse transmission route (Rajão et al., 2017). The most obvious example of IAV interspecies transmission occurred in 2009 pandemic, when H1N1pdm09 was identified as the causative agent of respiratory disease outbreaks in swine, after reports of outbreaks related to the same virus in human population (Dawood et al., 2009; Vincent et al., 2014). Biosafety measures on farms such as entry control, removal of sick workers and vaccination of workers are important to prevent IAV human to swine transmission. In addition, biosecurity measures can also minimize the risks to human health, since transmission from pig to man may also occur. Monitoring influenza subtypes infection in the human population is a tool that may help foresee possible outbreaks of influenza in the swine.

To differentiate the H1Delta samples between H1N1Delta or H1N2Delta a subtyping PCR for NA glycoprotein was performed. It was possible to subtype all samples from 2012-2015. However, in 2017-2018, 82% of the samples were not subtyped. The positive controls, for both N1 and N2 reactions amplified as expected, indicating that the reaction was performed correctly. So, the high percentage of non-subtyped samples in 2017-2018 was intriguing and may be an indicative of pointed mutations in the primers sites on NA gene that could have occurred in the last two years, resulting in failure of RT-PCR detection. The primes used for NA subtyping were previously described by Choi et al. (2002) and designed using sequences of reference strains of H1N1 and H3N2 from 1976 and 1998, respectively, both isolated in United States. The alignment of these primers with sequences of Brazilian SIV from 2009-2013 demonstrated 77% -100% of similarity, and the lowest value (77%) was related to a primer for

the detection of N1. Even with this dissimilarity, many samples were subtyped for N1 gene. However, differences between North and South American samples may be present, mostly from more recent years (2017-2018). The variability of HA protein has been further studied and reports related to antigenic variability of NA are rare. However, changes in this glycoprotein are known to occur (Air and Laver, 1986; Shil, 2011). Further studies to analyze the full length of NA gene must be done to confirm the differences and designing primers for N detection from Brazilian sequences could be a valuable tool to rapidly differentiate H1Delta samples by PCR.

A large majority of N1-positive samples were co-infected with H1N1pdm09. Since there was no sequencing of the RT-PCR products, it is unknown whether N1 gene was related to the H1N1Delta or H1N1pdm09 subtype. Co-infected samples may generate a false-positive H1Delta subtyping result, especially in samples co-infected with H1N1pdm09 and H3N2, since the primers used in the study may detect NAs of different subtypes. In this case the application of these primers for N1 subtyping is only possible in samples without co-infection.

From positive samples for N1, only one, from 2012-2013, was not co-infected with H1N1pdm09. Previously studies have detected H1N1Delta in nasal swab of swine from Santa Catarina state in 2014 (Schaefer et al., 2017). This highlights the presence of this lineage in Brazil over the last years, being necessary more studies related to its detection and characterization by complete genome sequencing, in order to generate more data about its circulation and presence in Brazilian swine population.

The phylogenetic analyses within each SIV subtype was performed by pairwise distance, estimated by p-distance method. It was observed a similarity of 88-100%, 75-100% and 91-100% within sequences of H1N1pdm09, H1Delta and H3N2, respectively (Supplementary Table 6, 7 and 8). The p-distance variation within sequences of each subtype was due to the differences found between them, mainly related to the period of occurrence.

The analyses of partial sequences of HA H1N1pdm09 indicated a difference between the samples that circulated from 2012-2015 and 2017-2018. Most of the 2012/2013 samples have grouped into a single group (Group B) with a high similarity within each other, but lower similarity between sequences from 2009-2010 samples, highlighting that samples from Group B already differed from previous years. Samples from 2014/2015 divided into 2 groups (Group A and C), with a difference of 11% between them, indicating that in the same period, different strains may have circulated in the swine population. Among the two groups, Group C was the

one that most differed from the 2009-2010 samples, suggesting that drift events may have occurred. It is interesting to note that samples from cluster C were the ones that had the highest number of changes in amino acid residues (Figure 10), especially in antigenic sites of the HA protein. When comparing Group A and C with the 2012-2013 samples (Group B), a similarity of 96-98% and 89-90%, respectively, was found. These data demonstrate that 2014-2015 samples have differed from previous years samples.

Differences in similarity between H1N1pdm09 partial sequences suggest that this virus has been evolving in Brazil since 2009. This evolution may be associated with point mutations in the HA sequence, that may have occurred due to immune pressure in swine and even in human population, as it was observed a high prevalence of H1N1pdm09 in farms and in human population for many years.

The amino acid sequence alignment between SIV H1N1pdm09 detected in Brazil from 2009 and 2010, human and swine vaccine strains and the partial HA sequences obtained from H1N1pdm09 viruses described in this study allowed the identification of 3 antigenic sites of HA: Cb, Sa and Ca2. They are located mainly in the globular region of HA1 and are highly subject to variation. Amino acid variation in these regions is due to the high viral replication rate and RNA polymerase reading errors during virus replication (Vries, 2013). Antigenic sites are subject to antibody neutralization and amino acid substitution in these regions contributes for viruses escaping from the immune response induced by an infection or vaccination (Sriwilaijaroen and Suzuki, 2012).

The only vaccine available in Brazil against SIV, licensed in 2014, has the A/CA/4/2009 (H1N1) strain in its composition. Samples from 2009-2010 presented amino acid differences in relation to the vaccine virus. At residue 129, which belongs to an antigenic site, it is noted that in the vaccine strain it is present Arg, whereas in four sequences from 2012-2013 it is composed by Lys (Figure 10). Still analyzing the vaccine strain, at residues 78, 144 and 145 belonging also to antigenic sites, the amino acids Ile, Asp and Ser are present, respectively. However, in these three sites, Leu, Glu and Thr, respectively, are present in six sequences from 2014-2015 (Figure 10). Punctual variations in other residues (53, 71, 86, 88, 99 and 113) could also be observed in samples from 2012-2015 (Figure 10). The data suggests that H1N1pdm09 virus may have evolved over the years and possible changes in immunologically important epitopes may have occurred.

At residues 100 and 101, from the analyzed sequences, the amino acids Ser and Asn, respectively, could be observed in sequences from 2009-2015, but not in human vaccine strain of 2016. However, these amino acids could be found in 2017-2019 human vaccine strains. This may indicate that variations observed in SIV and human IAVs can be observed over time in the both species and can be important to species, being important the monitoring IAVs in swine and humans. The epidemiological surveillance of human influenza in the southern and northern hemisphere is vigorously conducted by WHO, in order to recommend vaccine strains to be included in human vaccines for the next year. In 2016, A/California/7/2009(H1N1) vaccine strain pdm09 was used in human vaccination campaigns. In the same year, from the monitoring and genetic characterization of circulating IAVs, it was observed an antigenic variation of H1N1pdm09 which was different from the vaccine strain. Thus, in the following year, A/California/7/2009(H1N1) pdm09 was replaced by A/Michigan/45/2015(H1N1) pdm09 strain which was also recommended for 2018 and 2019 (WHO, 2018). These data show that surveillance of SIV is important in Brazil to monitoring variation which could help establish updates for swine vaccine production and also to compare genetic characterization data of SIV and human IAV.

Sequenced H1Delta samples were divided into 2 groups (A and B). It is interesting to note that group A samples were mostly from 2014/2015 and B from 2017/2018, and these two groups had similarity of 85-92%, suggesting that even in a partial sequence of H1Delta, there may be differences in samples from different times. However, similarities can also be found, since for each group one sample from 2012/2013 was present. Differences between these samples in the distinct periods, could be better observed if the sampling number were higher.

Variations between the two H1Delta groups (A and B) comparing to human seasonal H1N1 and swine and wild boar H1N2 samples, highlight the importance of further studies to analyze complete sequences of H1Delta samples in Brazil. Previous detection of H1N1Delta, from human seasonal origin in swine in Brazil (Schaefer et al., 2017) has been reported, however few data about its characterization and occurrence in the country is available.

It was found that from 2012-2018, H3N2 samples from group A and Cat2 had a high similarity with H3N2 swine North American and human sequences from 1998-2018, previously deposited at Genbank. Cat2 sample was distant from group A in the phylogenetic tree because it showed high similarity also with samples of North American swine H3N2 (99-100%), but by

p-distance the sample is also very similar to group A (99-100%), and therefore cannot consider that the sample is dissimilar to group A by analyzing only the tree. All the sequences from group A, Cat 2, North American swine and human sequences from 1998-2018 have demonstrated 7-10% dissimilarity with SarH3 and Brazilian swine H3N2 sequences from 2011. It can be inferred that H3 samples do not vary significantly over time, unlike the H1N1pdm09 samples. This may be associated with low immune pressure imposed on the subtype, as before 2017 there was a higher prevalence of H1N1pdm09, and also in Brazil there is no vaccination against H3N2.

There are few studies about genetic characterization of circulating swine influenza virus in Brazilian herds. In order to have a more consistent data and a more accurate analyses, further studies analyzing the complete HA gene of the subtypes circulating in Brazil from different years, previous and after the introduction of vaccination must be performed. The genetic characterization of complete HA of swine subtypes could also possibility studies comparing the human and swine circulating strains. This analysis would make it possible to assess amino acid changes that could lead to alteration of the conformation of viral epitopes, which antibodies induced by the vaccine would be incapable of neutralizing, and is essential to better understand the evolution of the virus in the country and may be used as a tool in the development of vaccines that provide protection to Brazilian herds.

7. Conclusion

- It was possible to standardize the RT-PCR technique, which demonstrated high sensitivity and specificity and in addition for being a rapid test, its use is recommended for subtyping SIV in field samples.

- From the analysis of the routine samples, it was possible to observe that there is a circulation of 4 strains of SIV in Brazil.

- The occurrence of different subtypes, demonstrated both by antigen detection and serology, varied over time with H3N2 being the most observed in the last two years. It is suggested that H1N1pdm09 viruses evolved over time in Brazil, but H3N2 did not suffered significant variations.

- The amino acid residue analysis of the H1N1pdm09 sequences have demonstrated point substitutions over the years, suggesting a possible occurrence of changes in immunologically important epitopes.

- Detection studies and genetic characterization of SIV are extremely important for the epidemiological monitoring of the virus in Brazil and also for the elaboration of effective vaccines.

8. References

AIR, G. M.; LAVER, W. G. The molecular basis of antigenic variation in influenza virus. Advances in Virus Research, v. 31, n. C, p. 53–102, 1986.

Associação Brasileira de Proteína Animal. *Relatório Anual 2017*. Available at: http://abpabr.com.br/storage/files/3678c_final_abpa_relatorio_anual_2016_portugues_web_reduzido.pdf Accessed on January 11, 2019.

BARCELLOS, D. E. S. N.; BOROWSKI, S. M. ; GHELLER, N. B. ; SANTI, M. ; MORES, T. J. Relação entre ambiente e doenças respiratórias em suínos.. *Acta Scientiae Veterinariae*, v. 36, p. 87-94, 2008.

BEAN, B.; MOORE, B.M. STERNER, B. ET AL. Survival of influenza viruses on environmental surfaces. Journal of Infectious Diseases, v.146, p. 47-51, 1982.

BEARD, C. W. Serologic procedures. In: *Isolation and identification of avian pathogens*. S. B. Hitchner, C. H. Domermuth, H. G. Purchase, and J. E. Williams, eds. American Association of Avian Pathologists, College Station, Texas. pp. 129-135, 1980.

BIONDO, N.; SCHAEFER, R.; GAVA, D. et al. Genomic analysis of influenza A virus from captive wild boars in Brazil reveals a human-like H1N2 influenza vírus. *Vet. Microb*., v. 168 (1), p. 34-40, 2014.

BOUVIER, N. M.; PALESE, P. The biology of influenza viruses. *NIH Public Access*. v. 26, n. Suppl 4, 2011.

BRASIL (2014). Ministério da Agricultura Pecuária e Abastecimento. Departamento de fiscalização de insumos pecuários coordenação de fiscalização de produtos veterinários. ATO Nº 2, DE 6 DE MARÇO DE 2014. Brasília, 2014. Available at: http://www.cidasc.sc.gov.br/defesasanitariavegetal/files/2018/08/Instru%C3%A7%C3%A3o-Normativa-n%C2%BA5-2014-Vazio-sanit%C3%A1rio-soja.pdf. Accessed on January 29,2019.

BRENTANO, L.; CIACCI-ZANELLA, J. R.; MORES, N. et al. Levantamento Soroepidemiológico para Coronavírus Respiratório e da Gastroenterite Transmissível e dos Vírus de Influenza H3N2 E H1N1 em Rebanhos Suínos no Brasil. In: COMUNICADO TÉCNICO, 306. 2002, Concórdia. Comunicado Técnico... Concórdia: Embrapa Suínos e Aves 2002.

BROCKMEIER, S.L.; HALBUR, P.G.; THACKER, E.L. Porcine respiratory disease complex. In *Polymicrobial Diseases*, ed. KA Brogden, JM Guthmiller, pp. 231–58, 2002.

BROWN, I. H. The epidemiology and evolution of influenza viruses in pigs. *Vet.Microb*. v. 74, p.29-46, 2000.

BROWN, I. H., DONE, S.H.; SPENCER, Y.I. et al. Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains. *Vet. Rec*., v. 132, p. 598- 602, 1993.

CARRAT, F.; FLAHAULT, A. Influenza vaccine: The challenge of antigenic drift. Vaccine, v. 25, n. 39–40, p. 6852–6862, 2007

CARR, J., WILLIAMS, D. G., & HAYDEN, R. T. Molecular Detection of Multiple Respiratory Viruses. *Molecular Diagnostics,* 289–300, 2000.

CARON, L.F.; JOINEAU, M.E.G.; SANTIN, E. et al. Seroprevalence of H3N2 influenza a virus in pigs from Paraná (South Brazil): interference of the animal management and climatic conditions. Virus Rev. Res., v. 15, n.1, 2010.

CIACCI-ZANELLA J.R., SCHAEFER R., DOS REIS J.K., GUEDES R.M., LOBATO Z.I., LEITE R.C. Serological evidence of swine influenza in Brazil. *Influenza Other Respir Viruses*, 7(2):109-12, 2013.

CIACCI-ZANELLA, J.R, et al. Prevalence of influenza viruses infection in swine herds in Brazil in 2011. In: 22nd International Pig Veterinary Society Congress, Jeju, Korea, . p. 32–34, 2012.

CIACCI-ZANELLA, J. R. et al. Influenza A virus infection in Brazilian swine herds following the introduction of pandemic 2009 H1N1. Veterinary Microbiology, v. 180, n. 1–2, p. 118–122, 2015.

CHARLEY, B.; RIFFAULT, S.; VAN REETH, K. Porcine innate and adaptative immune responses to influenza and coronavirus infections. Annals of the New York Academy of Sciences, v. 1081, p. 130–136, 2006.

CHAUHAN, N. et al. Laboratory diagnosis of swine flu : a review. *Artificial Cells, Nanomedicine, and Biotechnology*, p. 189–195, 2012.

CHEN, X.; LIU, S.; GORAYA, M.U.; MAAROUF, M.; HUANG, S.; CHEN J.L. Host immune response to influenza A virus infection. *Front Immunol*., v. 9, p. 1–13, 2018.

CHEN, Q.; MADSON, D.; MILLER, C.L.; HARRIS, D.L. Vaccine development for protecting swine against influenza virus. *Animal Health Res Rev*., Dec; 13(2), p. 181-95, 2012.

CHEN W, CALVO PA, MALIDE D, GIBBS J, SCHUBERT U, BACIK I, ET AL. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med*. 2001 Dec; 7(12):1306–12.

CHOI, Y. K. et al. Detection and subtyping of swine influenza H1N1 , H1N2 and H3N2 viruses in clinical samples using two multiplex RT-PCR assays. v. 102, p. 53–59, 2002.

CHOTHE S.K, et al. Avian and human influenza virus compatible sialic acid receptors in little brown bats. *Sci Rep*. 7(1):660, 2017

COLMAN, P. M.; HOYNE, P. A.; LAWRENCE1, A. M. C. Sequence and Structure Alignment of Paramyxovirus Hemagglutinin-Neuraminidase with Influenza Virus Neuraminidase. Journal of Virology, v. 67, n. 6, p. 2972–2980, 1993.

COSTA, E.A.; SILVEIRA, E.M.; GUEDES, M.I.M.C; DIAS, A.S.; ROSA, J.C.C; REHFELD, I.S.; MATOS, A.C.D; LOBATO, Z.I.P. Genetic characterization of influenza virus isolated from commercial herds pigs in southern and southeastern Brazil in 2014. In: XXVI Congresso de Virologia, 11 a 14 de Outubro, Costão do Santinho, Florianópolis, SC, 2015

COX, R. J.; BROKSTAD, K. A.; OGRA, P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand. *J. Immunol*., v. 59, p. 1-15, 2004.

CRISCI, E. et al. Review : Influenza virus in pigs. *Molecular Immunology*, v. 55, n. 3–4, p. 200–211, 2013.

CUNHA, R.G.; VINHA, V.R.; PASSOS, W.D. Isolation of a strain of Myxovirus influenzae-A suis from swine slaughtered in Rio de Janeiro. *Rev. Bras. Biol*., v.38., p. 13 – 17, 1978.

DAWOOD, F. S., JAIN, S.; FINELLI, L.; SHAW, M. W.; LINDSTROM, S.; GARTEN, R. J.; GUBAREVA, L. V.; XU, X.; BRIDGES, C. B.; UYEKY, T. M. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N. Engl. J. Med*., 360, 2605–2615, 2009.

Dee, S.A. Swine influenza. In: Kahn CM, Line S, Aiello SE, editors. *The Merck veterinary manual.* Whitehouse Station, NJ: Merck and Co; 2015. Available at: http://www.merckmanuals.com/mvm/respiratory_system/respiratory_diseases_of_pigs/swine_influenza.html. Accessed 11 Jan 2019.

DE JONG, J. C., et al. Antigenic drift in swine influenza H3 haemagglutinins with implications for vaccination policy. *Vaccine*., v. 17, p. 1321–1328, 1999.

De Jong MD, Bach VC, Phan TQ, Vo MH, Tran TT, Nguyen BH, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N Engl J Med*.352:686–9,2005.

DE VLEESCHAUWER, A. et al. Comparative pathogenesis of an avian H5N2 and a swine H1N1 influenza virus in pigs. *Plos One*, v. 4, n. 8, p. 1–10, 2009.

DETMER, S.E.; PATNAYAK, D.P.; JIANG, Y.; GRAMER, M.R.; GOYAL, S.M. Detection of Influenza a Virus in Porcine Oral Fluid Samples. *J Vet Diagn Invest*, v. 23, p. 241 – 247, 2011.

DIAS, A.; E, COSTA.; RAJÃO, D.; GUEDES, R.M.C.; ZANELLA, J.R.C.; LOBATO, Z.I.P. Distribution of antibodies against influenza virus in pigs from farrow-to-finish farms in Minas Gerais state, Brazil. *Influenza and other Respiratory Viruses*, v. 9, n. 3, p. 161–167, 2015.

DUCATEZ, M. F., et al. Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. *Emerg. Infect. Dis*. v.17, p. 1624–1629, 2011.

EASTERDAY, B.C.; VAN REETH, K. Swine Influenza. In: STRAW, B. E.; D'ALLAIRE, S.;

ENKIRCH,T., VON MESSLING, V. Ferret models of viral pathogenesis. *Virology*. 479-480*,* 259–70. 2015

MENGELIN, W.L.; TAYLOR, D. J. (Ed). *Diseases of swine*. 8th ed. Ames: Iowa State University Press, p. 277-290, 1999.

FAO. Food and Agriculture Organization of the United Nations /World Organisation for Animal Health/World Bank. *Good practices for biosecurity in the pig sector – Issues and options in developing and transition countries*. FAO Animal Production and Health Paper No. 169. Rome, 2010.

FLORES, E.F.; Lovato, L.T.; Silva, M.S.; Dezengrini, R.; Diel, D. Virologia Veterinária: Orthomyxoviridae. Ed. 1. Santa Maria: *UFSM*, 2007. p. 723-733; 738-742, 2007.

FLYNN, O. et al. Influenza D Virus in Cattle, Ireland. *Emerging Infectious Diseases* v. 24, n. 2, p. 2016–2018, 2018.

FRAIHA, A., et al. Estudos sorológicos dos subtipos do vírus influenza a suíno circulantes nas regiões sul e sudeste do brasil no período de 2014 a 2015. XVIII Congresso Nacional da ABRAVES, Goiânia, 2017

GAMBLIN, S. J.; SKEHEL, J. J. Influenza hemagglutinin and neuraminidase membrane glycoproteins. *Journal of Biological Chemistry*, v. 285, n. 37, p. 28403–28409, 2010.

GAUGER, P.C.; VINCENT, A.L.; LOVING, C.L.; LAGER, K.M.; JANKE, B.H.; et al. Kinetics of lung lesion development and pro-inflamatory cytokine response in pigs with vaccine-associated enhanced respiratry disease induced by challenge with pandemic (2009) A/H1N1 influenza vírus. *Vet. Pathol*., v. 49 (6), 2012

GARTEN, R. J., et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science*., v. 325, p.197–201, 2009.

GAVA,D, et al. Otimização de RT-PCR multiplex para subtipagem do vírus influenza A. Congresso Brasileiro de Veterinários Especialistas em Suínos. Fortaleza, Abraves, 2011.

GUAN, Y., SHORTRIDGE, K. F.; KRAUSS, S.; LI, P. H.; KAWAOKA, Y.; WEBSTER, R. G. Emergence of avian H1N1 influenza viruses in pigs in China. *J. Virol*., v. 70, p. 8041–8046, 1996.

HAACH, V., GAVA, D.CANTÃO, M., SCHAEFER, R. Multiplex RT-PCR assay for subtyping of swine influenza virus in Brazil. XXIX Brazilian Congress of Virology & XIII Mercosur Meeting of Virology. 2018

HALL, T.A. BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, v. 41, p. 95-98, 1999.

HEROLD, S., et al. Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. *J. Exp. Med., v.* 205, p. 3065–3077, 2008.

HOWARD, W.A.; ESSEN, S.C.; STRUGNELL, B.W.; RUSSELL, C.; BARRASS, L.; REID, S.M.; BROWN, I.H. Reassortant pandemic (H1N1) 2009 virus in pigs United Kingdom. *Emerg*

Infect Dis. v. 17(6), p. 1049–1052, 2011.

HUSSAIN, A.I.; CORDEIRO, M.; SEVILLA, E.; LIU, J. Comparison of egg and high yielding MDCK cell-derived live attenuated influenza virus for commercial production of trivalent influenza vaccine: in vitro cell susceptibility and influenza virus replication kinetics in permissive and semi- -permissive cells. *Vaccine., v.* 28 (22), p. 3848-3855, 2010

ICTV (International Committee on Taxonomy of Viruses), 2014. Virus Taxonomy: 2014 Release. Available at http://ictvonline.org/virusTaxonomy.asp. Accessed on January 16, 2019.

JANKE, B.H. Diagnosis of swine influenza. *Swine Health Prod.*, v. 8(2), p.79-84, 2000.

JANKE, B.H. Clinicopathological features of swine influenza. Curr *Top Microbiol Immunol.,* v. 370, p. 69–83, 2013.

KHURANA S, LOVING CL, MANISCHEWITZ J, et al. Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease. Sci. Transl. Med. 2013.

KILLIP, M. J., FODOR, E., & RANDALL, R. E. Influenza virus activation of the interferon system. *Virus Research,* v. 209, p.11–22, 2015.

KITIKOON, P. et al. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. *Veterinary Immunology and Immunopathology*, v. 112, p. 117–128, 2006.

KOCER, Z. A., OBENAUER, J., ZARAKET, H., ZHANG, J., REHG, J. E., RUSSELL, C. J., & WEBSTER, R. G. Fecal Influenza in Mammals: Selection of Novel Variants. *Journal of Virology*, v. 87(21), p.11476–11486, 2013.

KOEN, J.S. A practical method for field diagnosis of swine diseases. Am. *J. Vet. Med*., v.14, p.468-70, 1919.

KOTHALAWALA, H.; TOUSSAINT, M.J.; GRUYS, E. An overview of swine influenza. *Vet. Q*., v. 28, p. 46-53, 2006

KOWALCZYK, A.; MARKOWSKA-DANIEL, I. Detection and subtyping of swine influenza viruses in clinical samples by the mean of developed multiplex polymerase chain reaction assays. *Bull Vet Inst Pulawy* 53, 319-325, 2009.

KRAMMER, F., SMITH, G.J.D., FOUCHIER, R.A.M. *et al.* Influenza. *Nat Rev Dis Primers* **4,** 3, 2018.

KREIJTZ, J.H.C.M.; FOUCHIER, R.A.M.; RIMMELZWAAN, G.F. Immune response to influenza vírus infection. Virus Research, v. 162, p. 19- 30, 2011.

KUMAR, S.; STECHER, G.; TAMURA, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Molecular. Biology and Evolution*,33, p. 1870-1874, 2016.

KYRIAKIS, C. S., I. H. BROWN, E. FONI, G. KUNTZ-SIMON, J. MALDONADO, F. MADEC, S. C. ESSEN, C. CHIAPPONI, and R. K. VAN, Virological surveillance and preliminary antigenic characterization of influenza viruses in pigs in five European countries from 2006 to 2008. Zoonoses Public Health 58, 93–101, 2011.

LAMB, R.A.; KRUG, R.M. Orthomyxoviridae: the viruses and their replication. In: KNIPE, D.M.; HOWLEY, P.M.; GRIFFIN, D.E.; LAMB, R.A.; MARTIN, M.A.; ROIZMAN, B.; STRAUS, S.E. and Eds. *Fields Virology*, 4th edition, Philadelphia: Lippincott Williams & Wilkins, p. 1216–1253, 2001.

LANDOLT, G. A.; OLSEN, C. W. Up to new tricks - a review of cross-species transmission of influenza A viruses. *Animal health research reviews / Conference of Research Workers in Animal Diseases*, v. 8, n. 1, p. 1–21, 2007.

LANDOLT, G.A.; KARASIN, A.I.; PHILLIPS, L. et al. Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. *J Clin Microbiol*., v. 41., p. 1936–1941, 2003.

LEUWERKE, B. et al. Comparison of three serological assays to determine the cross-reactivity of antibodies from eight genetically diverse U . S . *Swine influenza viruses*. v. 432, p. 426–432, 2008.

LOEFFEN, W.L.A.; HEINEN, P.P.; BIANCHI, A.T.J. et al. Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus. Vet. *Immunol. Immunopathol*., v. 92, p. 23-35, 2003

LOEFFEN, W.L.A.; HUNNEMAN, W.A.; QUAK, J. et al. Population dynamics of swine influenza virus in farrow-to-finish and specialized finishing herds in the Netherlands. *Vet. Microbiol*., v. 137, p.45–50, 2009.

LORUSSO, A.; VINCENT, A. L.; HARLAND, M. L. et al. Genetic and antigenic characterization of H1 influenza viruses from United States swine from 2008. *J. Gen. Virol*., v. 92, p. 919–930, 2011.

LYOO, K. S. et al. Comparative pathology of pigs infected with Korean H1N1, H1N2, or H3N2 swine influenza A viruses. *Virology Journal*, v. 11, n. 1, p. 1–6, 2014.

MA, W.; RICHT, J. A. Swine influenza vaccines: current status and future perspectives. *Anim. Health Res. Rev*., v. 11, p. 81–96, 2010.

MAES, D.; SEGALES, J.; MEYNS, T.; SIBILA, M.; PIETERS, M.; HAESEBROUCK, F. Control of Mycoplasma hyopneumoniae infections in pigs.*Veterinary Microbiology*, v. 126, n. 4, p. 297–309, 2008.

MANCINI, D. A. P.; CUNHA, E. M. S.; MENDONÇA, R. M. Z. Evidence of swine respiratory infection by influenza viruses in Brazil. *Virus Rev. Res*., v. 11, p. 39-43, 2006.

MAHER J. A. AND DESTEFANO, J.The ferret: an animal model to study influenza virus. *Lab. Anim. (NY)* **33**, 50–53, 2004.

MAHONY, J. B. Detection of Respiratory Viruses by Molecular Methods. *Clinical Microbiology Reviews*., v. 21, n. 4, p. 716–747, 2008.

MARTINS, N.R.S. An Overview on Avian Influenza. Brazilian Journal of Poultry Science, 14, 71-158, 2012.

MUKHERJEE, A. et al. Genetic characterization of circulating 2015 A(H1N1)pdm09 influenza viruses from Eastern India. *PloS One*, v. 11, n. 12, p. 1–14, 2016.

NELSON, M. I. et al. Global transmission of influenza viruses from humans to swine. Journal of General Virology, v. 93, n. PART 10, p. 2195–2203, 2012.

NELSON, M. I. et al. Influenza A Viruses of Human Origin in Swine, Brazil. Emerging Infectious Diseases, v. 21, n. 8, p. 1339–1347, 2015.

OLSEN, C. W. et al. Triple reassortant H3N2 influenza A viruses, Canada, 2005. *Emerg. Infect. Dis*., v. 12 (7), p.1132–1135, 2006

OIE. World Organization for Animal Health . Manual of diagnostic tests and vaccines for terrestrial animals. Paris; OIE; 2015. Swine influenza. Available at: http://www.oie.int/standard-setting/terrestrial-manual/access-online/ . Accessed on January 29,2019.

PASCUA, P. N. Q. et al. Seroprevalence and genetic evolutions of swine influenza viruses under vaccination pressure in Korean swine herds. *Virus Research*, v. 138, n. 1–2, p. 43–49, 2008.

PEIRIS, J.S. et al. Co-circulation of avian H9N2 and contemporary ‘‘human’’ H3N2 influenza A viruses in pigs in southeastern China: Potential for genetic reassortment?. *J Virol*., v. 75, p. 9679–9686, 2001.

PEDERSEN, J. C. Hemagglutination-inhibition assay for influenza virus subtype identification and the detection and quantitation of serum antibodies to influenza virus. *Methods Mol. Biol*. 1161:11–25. 2014.

PIELAK, R. M.; CHOU, J. J. Influenza M2 proton channels. *Biochimica et Biophysica Acta - Biomembranes*, v. 1808, n. 2, p. 522–529, 2011.

PIFFER, I.A.; BRITO, J.R.F. Pneumonia em suínos. *Suinocultura Dinâmica*, Concórdia:Embrapa Suínos e Aves, a.II, n.8, p.6, 1993.

PORTELA, A.; DIGARD, P. The influenza virus nucleoprotein : a multifunctional RNA-binding protein pivotal to virus replication. *Journal of General Virology*, v. 83, n. 4, p. 723–734, 2002.

POTTER CW, OXFORD JS. Determinants of immunity to influenza infection in man. Br Med Bull,35:69-75, 1979.

RAJÃO, D.S.; WALLIA, R.R.; CAMPBELL, B.; GAUGER, P.C.; JANAS-MARTINDALE,

A.; KILLIAN, M.L.; VINCENT, A.L. Reassortant between swine H3N2 and 2009 pandemic H1N1 in the United States resulted in influenza A viruses with diverse genetic constellations with variable virulence in pigs. *J Virology*., v.91., e01763-16, 2017

RAJÃO, D.R; COSTA, A.T.R; BRASIL, B.S.F.A; DEL PUERTO, H.L; OLIVEIRA, F.G; ALVES, F; BRAZ, G.F; REIS, J.K.P; GUEDES, R.M.C; LOBATO, Z.I.P & LEITE, R.C. Genetic characterization of influenza virus circulating in Brazilian pigs during 2009 and 2010 reveals a high prevalence of the pandemic H1N1 subtype. *Influenza and Other Respiratory Viruses*, 7(5):783-90, 2013a.

RAJÃO D.S., ALVES F., DEL PUERTO H.L., BRAZ G.F., OLIVEIRA F.G., CIACCI-

Relova, D., Rios, L., Acevedo, A., Coronado, L., Perera, C., & Pérez, L. Impact of RNA Degradation on Viral Diagnosis: An Understated but Essential Step for the Successful Establishment of a Diagnosis Network. *Veterinary Sciences*, 5(1), 19, 2018.

REID, A.H.; FANNING, T.G.; HULTIN, J.V.; TAUBENBERGER, J.K.; Origin and evolution of the 1918 Spanish influenza virus hemagglutinin gene. *Proc. of Natl. Academy of Sciences USA*., v. 96, p. 1651–1656, 1999.

REID, A. H.; TAUBENBERGER, J. K. The origin of the 1918 pandemic influenza virus: A continuing enigma. *Journal of General Virology*, v. 84, n. 9, p. 2285–2292, 2003.

RESENDE, P.; BORN, P.; MATOS, A. et al. Whole-Genome Characterization of a Novel Human Influenza A(H1N2) Virus Variant, Brazil. *Emerging Infectious Diseases*., v. 23., p. 152-154, 2017.

RICHT, J. A.; LAGER, K. M.; JANKE, B. H. et al. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. *J. Clin. Microbiol.*, v. 41, p. 3198-3205, 2003.

SANDBULTE, M.R., SPICKLER, A.R., ZAABEL, P.K., ROTH, J.A. Optimal Use of Vaccines for Control of Influenza A Virus in Swine. *Vaccines* (Basel) 3, 22-73, 2015.

SCHAEFER, R.; GAVA, D.; HAACH,V.; CANTÃO, M.E.; NELSON, I.M. Novel human-like h1n1 swine influenza virus with the six internal genes derived from h1n1/2009 virus. In: XXVIII Congresso de Virologia, 6 a 10 de Outubro, Belo Horizonte, Minas Gerias, Brazil, 2015a.

SCHAEFER, R.; RECH, R.R.; GAVA, D. et al. A human-like H1N2 influenza virus detected during an outbreak of acute respiratory disease in swine in Brazil. *Arch. Virol.*, v. 160 (1), p. 29-38, 2015b.

SCHAEFER, R.; RECH, R.R.; SILVA, M.C.; GAVA, D.; CIACCI- ZANELA, J.R. Orientações para o diagnóstico de influenza em suínos. *Pesq. Vet. Bras.*, v. 33 (1), p. 61-73, 2013.

SCHAEFER, R., ZANELLA, J.R.C., BRENTANO, L., et al. Isolation and characterization of a pandemic H1N1 influenza virus in pigs in Brazil. *Pesq. Vet. Bras*., v. 31, p. 761-767, 2011

SHAO, W. et al. Evolution of Influenza A Virus by Mutation and Re-Assortment. *International Journal of Molecular Sciences*, v. 18(8), p.1650, 2017.

SHOPE, R.E. Swine influenza. III. Filtration experiments and aetiology. *J. Exp. Med*., v.54, p.373-380, 1931.

SONG, M. S. et al. Evidence of human-to-swine transmission of the pandemic (H1N1) 2009 influenza virus in South Korea. *Journal of Clinical Microbiology*, v. 48, n. 9, p. 3204–3211, 2010.

SRIWILAIJAROEN, N.; SUZUKI, Y. Molecular basis of the structure and function of H1 hemagglutinin of influenza virus. *Proceedings of the Japan Academy*, Series B, v. 88(6), p. 226–249. 2012.

SUN, X., et al. N-Linked Glycosylation of the Hemagglutinin Protein Influences Virulence and Antigenicity of the 1918 Pandemic and Seasonal H1N1 Influenza A Viruses. *Journal of Virology*, v. 87(15), p. 8756–8766, 2013.

TAMURA, K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Molecular Biology and Evolution*., v **9**, p. 678–687,1992.

TAKEMAE, N. et al. Genetic diversity of swine influenza viruses isolated from pigs during 2000 to 2005 in Thailand. *Influenza and other Respiratory Viruses*, v. 2, n. 5, p. 181–189, 2008.

TAKEMAE, N. et al. Effect of herd size on subclinical infection of swine in Vietnam with influenza A viruses. *BMC Veterinary Research,* v. 12, n. 1, p. 1–11, 2016.

TEREBUH, P. et al. Transmission of influenza A viruses between pigs and people, Iowa, 2002 – 2004. *Influenza and Other Respiratory Viruses*, p. 387–396, 2010.

THOMPSON, J. D.; HIGGINS, D. G.; GIBSON, T. J. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, v. 22, p. 4673–4680, 1994.

TONG, S. et al. New World Bats Harbor Diverse Influenza A Viruses. *PLoS Pathogens*, v. 9, n. 10, 2013.

VAN REETH, K. Avian and swine influenza viruses: our current understanding of the zoonotic risk. *Vet. Res*., v. 38, p. 243–260, 2007.

VAN REETH, K.; BROWNB, I.; ESSEN, S.; PENSAERT, M. Genetic relationships, serological cross-reaction and cross-protection between H1N2 and other influenza A virus subtypes endemic in European pigs. *Virus Res*., v. 103, p. 115–124, 2004.

VAN REETH, K.; MA, W. Swine influenza virus vaccines – to change or not to change – That's the question. *Curr Top Micro Immu*, 370: 173–200, 2012.

VINCENT, A.L. et al. Review of influenza A virus in swine worldwide: a call for increased

surveillance and research. *Zoo. Publ. Health*, v. 61, p. 4- 17, 2014.

VINCENT, A. L. et al. Swine Influenza Viruses: A North American Perspective. In: MARAMOROSCH, K.; SHATKIN, A.J.; MURPHY, F. A. (Eds). *Advances in Virus Research*. Vol. 72. Burlington: Academic Press, p.127-154, 2008.

VOETEN, T. et al. Use of Recombinant Nucleoproteins in Enzyme-Linked Immunosorbent Assays for Detection of Virus-Specific Immunoglobulin A (IgA) and IgG Antibodies in Influenza Virus A- or B-Infected Patients. *Journal of clinical microbiology*., v. 36, p. 3527-31, 1999.

WEBER, T.P.; STILIANAKIS, N.I. "Inactivation of influenza A viruses in the environment and modes of transmission: a critical review". *Journal of Infection*, vol. 57, no. 5, pp. 361–373, 2008.

WEBBY, R. J.; ROSSOW, K.; ERICKSON, G. et al. Multiples lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. *Virus Res*., v. 103, p. 67-73, 2004.

WELSH, M. D. et al. Initial incursion of pandemic (H1N1) 2009 influenza A virus into European pigs. *Veterinary Record*, v. 166, n. 21, p. 642–645, 2010.

WEBSTER, R. G.; BEAN, W. J.; GORMAN, O. T. et al. Evolution and ecology of influenza A viruses. *Microbiol. Rev*., v. 56, p. 152-179, 1992.

WHITE, L. A.; TORREMORELL, M.; CRAFT, M. E. Influenza A virus in swine breeding herds: Combination of vaccination and biosecurity practices can reduce likelihood of endemic piglet reservoir. *Preventive Veterinary Medicine*, v. 138, p. 55–69, 2017.

WOOD, J.M.; GAINES-DA, S R.E.; TAYLOR, J.; CHAKRAVERTY, P. Comparison of influenza serological techniques by international collaborative study. *Vaccine*, v. 12, p. 167–174, 1994.

WHO. World Health Organization. Manual for the laboratory diagnosis and virological surveillance of influenza. 2011. Available at: https://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en/ . Accessed on January 29, 2019

WHO. World Health Organization. FluNet. Available from: http://www.who.int/influenza/gisrs_laboratory/flunet/en/. Acessed on January 29, 2019.

YOON SW.; WEBBY R.J.; WEBSTER R.G. Evolution and Ecology of Influenza A Viruses. *Current Topics in Microbiology and Immunology*, v 385, p. 359-375, 2014.

ZHANG, W.; EVANS, D. H. Detection and identification of human influenza viruses by the polymerase chain reaction. *Journal of Virological Methods.,* v. 33, p. 165–189, 1991.

ZHU, H. et al. History of Swine Influenza Viruses in Asia. *Current Topics in Microbiology and Immunology*, 57–68, 2011.

9. Supplementary Information

Supplementary Table 1. Reference sequences used in this study for the primer design.

HA Subtype	Strain designation	Subtype	HA gene Genbank accession no.
H1	(A/swine/Brazil/4/2009(H1N1)	H1N1pdm09	JQ666848.1
H1	(A/swine/Brazil/263/2012(H1N1))	H1N1pdm09	KM497003.1
H1	(A/swine/Brazil/18/2012(H1N1)	H1N1pdm09	KM496987.1
H1	(A/swine/Brazil/66/2011(H1N1)	H1N1pdm09	KM496995.1
H1	(A/swine/Brazil/132/2009(H1N1)	H1N1pdm09	KM497008.1
H1	(A/swine/Brazil/12A/2010(H1N1)	H1N1pdm09	JF421756.1
H1	(A/swine/Brazil/107-3A/2010(H1N1)	H1N1pdm09	KF683614.1
H1	(A/swine/Brazil/20/2010(H1N1)	H1N1pdm09	JQ666864.1
H1	(A/swine/Brazil/19/2010(H1N1)	H1N1pdm09	JQ666863.1
H1	(A/swine/Brazil/18/2010(H1N1)	H1N1pdm09	JQ666862.1
H1	(A/swine/Brazil/17/2009(H1N1)	H1N1pdm09	JQ666861.1
H1	(A/swine/Brazil/16/2009(H1N1)	H1N1pdm09	JQ666860.1
H1	(A/swine/Brazil/15/2009(H1N1)	H1N1pdm09	JQ666859.1
H1	(A/swine/Brazil/14/2009(H1N1)	H1N1pdm09	JQ666858.1
H1	(A/swine/Brazil/13/2009(H1N1)	H1N1pdm09	JQ666857.1
H1	(A/swine/Brazil/12/2009(H1N1)	H1N1pdm09	JQ666856.1
H1	(A/swine/Brazil/11/2009(H1N1)	H1N1pdm09	JQ666855.1
H1	A/swine/Brazil/10/2009(H1N1)	H1N1pdm09	JQ666854.1
H1	(A/swine/Brazil/9/2009(H1N1)	H1N1pdm09	JQ666853.1
H1	(A/swine/Brazil/G3P1/2013(H1N1)	H1N1pdm09	KP027601.1
H1	(A/swine/Brazil/8/2009(H1N1)	H1N1pdm09	JQ666852.1
H1	(A/swine/Brazil/31_11_1/2011(H1N2)	H1N2delta	KF680296.1
H1	(A/swine/Brazil/31_11_3/2011(H1N2)	H1N2delta	KF680291.1
H1	(A/swine/Brazil/232-11-14/2011(H1N2)	H1N2delta	KM507532.1
H1	(A/swine/Brazil/232-11-13/2011(H1N2)	H1N2delta	KM507530.1
H1	(A/swine/Brazil/185-11-7/2011(H1N2)	H1N2delta	KM507519.1
H1	(A/swine/Brazil/G2P1/2013(H1N2)	H1N2delta	KP027585.1
H1	(A/wild boar/Brazil/214-11-13D/2011(H1N2)	H1N2delta	KF572616.1
H3	(A/swine/Brazil/231-11-1/2011(H3N2)	H3N2	KM507535.1
H3	(A/swine/Brazil/355-11-6/2011(H3N2)	H3N2	KM507503.1
H3	(A/swine/Brazil/365-11-7/2011(H3N2)	H3N2	KM507511.1

Supplementary Table 2. Relation of subtyped and non-subtyped samples per state

Relation of Subtyped and Non-subtyped samples per State			
State	**Subtyped samples**	**Non-subtyped**	**Total per state**
RS	14	1	15
PR	13	8	21
SC	8	3	11
SP	1	1	2
MG	24	4	28
MS	1	0	1
GO	2	0	2
N/I	8	4	12
Total	**71**	**21**	**92**

Supplementary Table 3. Relation of detected subtypes per state and years.

Relation of identified subtypes per state and years							
2012/2013							
State	**H1N1 pdm09**	**H1Delta**	**H3N2**	**H1N1pdm09+ H1Delta**	**H1N1pdm09+ H3N2**	**H1N1pdm09+ H1Delta**	**H1N1pdm09+ H1Delta+H3N2**
RS	6	1	0	0	0	0	0
PR	1	1	0	0	0	0	0
SC	0	1	0	0	2	0	0
SP	0	0	0	0	0	0	0
MG	2	0	0	0	0	0	0
MS	0	0	0	0	0	0	0
GO	0	0	0	0	0	0	0
N/I	0	0	0	0	0	0	0
Total	9	3	0	0	2	0	0
2014/2015							
RS	7	0	0	0	0	0	0
PR	1	2	1	0	0	0	0
SC	4	0	0	0	0	0	0
SP	0	1	0	0	0	0	0
MG	9	1	0	2	0	0	0
MS	0	0	0	0	0	0	0
GO	0	0	0	0	0	0	0
N/I	6	0	0	1	0	0	0
Total	27	4	1	3	0	0	0
2017/2018							
RS	0	0	0	0	0	0	0
PR	2	1	0	0	1	0	0
SC	0	1	0	0	0	0	0
SP	0	0	0	0	0	0	0
MG	1	2	5	1	0	1	0
MS	0	1	0	0	0	0	0
GO	2	0	0	0	0	0	0
N/I	0	1	0	0	0	0	0
Total	5	6	5	1	1	1	3

Supplementary Table 4. Relation of sequenced samples per region and subtype

Relation of sequenced samples per year and subtype			
State	**2012/2013**	**2014/2015**	**2017/2018**
H1N1pdm09	4	19	0
H1Delta	3	4	6
H3N2	1	1	7
Total	**8**	**24**	**13**

Supplementary Table 5. Relation of sequenced samples per year and subtype

Relation of sequenced samples per region and subtype			
State	**H1N1pdm09**	**H1Delta**	**H3N2**
RS	7	1	0
PR	1	5	4
SC	3	2	1
SP	0	1	0
MG	8	4	4
MS	0	0	0
GO	0	0	0
N/I	4	0	0
Total	**23**	**13**	**9**

Supplementary Table 6. P-distance analysis of partial sequences of H1N1pdm09 subtype

	BRsw14/15pdm-Z12P	BRsw-14/15pdm-_Z15P	BRsw14/15pdm-_Z23P	BRsw14/15pdm-_Z32P	BRsw14/15pdm-_Z10P	BRsw14/15pdm-_Z18P	BRsw14/15pdm-_Z19P	BRsw14/15pdm-_Z27P	BRsw14/15pdm-_Z43P	BRsw14/15pdm-Z1P	BRsw14/15pdm_-_Z41P	BRsw12/13pdm-FLU8P	BRsw12/13pdm-FLU6P	BRsw12/13pdm-FLU7P	BRsw12/13pdm-FLU2P	BRsw14/15pdm-_Z9P	ZBRsw14/15pdm-_Z45P	BRsw14/15pdm-_Z35P	BRsw14/15pdm-_Z39P	BRsw14/15pdm-_Z12.2P	BRsw14/15pdm-Z26P	BRsw12/13pdm-FLU5P	BRsw12/13pdm_-_FLU17P	BRsw09pdm-JQ666845_A/swine/Brazil/1/2009_2009/08/11	BRsw09pdm-JQ666846_A/swine/Brazil/2/2009_2009/08/11	BRsw09pdm-JQ666847_A/swine/Brazil/3/2009_2009/08/11	BRsw09pdm-JQ666848_A/swine/Brazil/4/2009_2009/08/21	BRsw09pdm-JQ666849_A/swine/Brazil/5/2009_2009/08/28	BRsw09pdm-JQ666850_A/swine/Brazil/6/2009_2009/08/28	BRsw09pdm-JQ666851_A/swine/Brazil/7/2009_2009/09/26	BRsw09pdm-JQ666852_A/swine/Brazil/8/2009_2009/10/05	BRsw09pdm-JQ666853_A/swine/Brazil/9/2009_2009/10/05	BRsw09pdm-JQ666854_A/swine/Brazil/10/2009_2009/10/05	BRsw09pdm-JQ666855_A/swine/Brazil/11/2009_2009/10/18	BRsw09pdm-JQ666856_A/swine/Brazil/12/2009_2009/10/25	BRsw09pdm-JQ666857_A/swine/Brazil/13/2009_2009/11/10	BRsw09pdm-JQ666858_A/swine/Brazil/14/2009_2009/12/04	BRsw09pdm-JQ666859_A/swine/Brazil/15/2009_2009/12/21	BRsw09pdm-JQ666861_A/swine/Brazil/17/2009_2009/08/01	BRsw09pdm-JQ666860_A/swine/Brazil/16/2009_2009/08/01	BRsw09pdm-KM497008_A/swine/Brazil/132/2009_2009/09/09	BRsw10pdm-JF421756_A/swine/Brazil/12A/2010_2010/02/02	BRsw10pdm-JQ666862_A/swine/Brazil/18/2010_2010/05/11	BRsw10pdm-JQ666863_A/swine/Brazil/19/2010_2010/09/09	BRsw10pdm-JQ666864_A/swine/Brazil/20/2010_2010/09/09_	BRsw10pdm-KF683614_A/swine/Brazil/107-3A/2010_2010/07/14	2017/18/19-KY117023.1_(A/Michigan/45/2015(H1N1))	2010/16-KU933485.1_Influenza_A_virus_(A/California/07/2009(H1N1))	H1N1pdm_A-CA-4-2009_
BRsw14/15pdm-Z12P	1,000
BRsw-14/15pdm-_Z15P	1,000	1,000
BRsw14/15pdm-_Z23P	1,000	1,000	1,000
BRsw14/15pdm-_Z32P	1,000	1,000	1,000	1,000
BRsw14/15pdm-_Z10P	1,000	1,000	1,000	1,000	1,000
BRsw14/15pdm-_Z18P	1,000	1,000	1,000	1,000	1,000	1,000
BRsw14/15pdm-_Z19P	1,000	1,000	1,000	1,000	1,000	1,000	1,000
BRsw14/15pdm-_Z27P	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
BRsw14/15pdm-_Z43P	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
BRsw14/15pdm-Z1P	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
BRsw14/15pdm_-_Z41P	0,997	0,997	0,997	0,997	0,997	0,997	0,997	0,997	0,997	0,997	1,000
BRsw12/13pdm-FLU8P	0,955	0,955	0,955	0,955	0,955	0,955	0,955	0,955	0,955	0,955	0,955	1,000
BRsw12/13pdm-FLU6P	0,963	0,963	0,963	0,963	0,963	0,963	0,963	0,963	0,963	0,963	0,960	0,996	1,000
BRsw12/13pdm-FLU7P	0,961	0,961	0,961	0,961	0,961	0,961	0,961	0,961	0,961	0,961	0,960	0,996	1,000	1,000
BRsw12/13pdm-FLU2P	0,961	0,961	0,961	0,961	0,961	0,961	0,961	0,961	0,961	0,961	0,960	0,996	1,000	1,000	1,000
BRsw14/15pdm-_Z9P	0,881	0,881	0,881	0,881	0,881	0,881	0,881	0,881	0,881	0,880	0,873	0,867	0,881	0,880	0,880	1,000
ZBRsw14/15pdm-_Z45P	0,878	0,878	0,878	0,878	0,878	0,878	0,878	0,878	0,878	0,877	0,878	0,862	0,877	0,877	0,877	0,997	1,000
BRsw14/15pdm-_Z35P	0,875	0,875	0,875	0,875	0,875	0,875	0,875	0,875	0,875	0,875	0,875	0,861	0,875	0,875	0,875	0,994	0,997	1,000
BRsw14/15pdm-_Z39P	0,877	0,877	0,877	0,877	0,877	0,877	0,877	0,877	0,877	0,876	0,869	0,862	0,877	0,876	0,876	0,997	1,000	0,997	1,000
BRsw14/15pdm-_Z12.2P	0,877	0,877	0,877	0,877	0,877	0,877	0,877	0,877	0,877	0,876	0,869	0,862	0,877	0,876	0,876	0,997	1,000	0,997	1,000	1,000
BRsw14/15pdm-Z26P	0,877	0,877	0,877	0,877	0,877	0,877	0,877	0,877	0,877	0,876	0,869	0,862	0,877	0,876	0,876	0,997	1,000	0,997	1,000	1,000	1,000
BRsw12/13pdm-FLU5P	0,983	0,983	0,983	0,983	0,983	0,983	0,983	0,983	0,983	0,983	0,983	0,959	0,965	0,965	0,965	0,884	0,880	0,883	0,880	0,880	0,880	1,000
BRsw12/13pdm_-_FLU17P	0,973	0,973	0,973	0,973	0,973	0,973	0,973	0,973	0,973	0,973	0,969	0,955	0,963	0,964	0,964	0,891	0,889	0,887	0,887	0,887	0,887	0,972	1,000
BRsw09pdm-JQ666845_A/swine/Brazil/1/2009_2009/08/11	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,988	0,967	0,973	0,970	0,970	0,891	0,889	0,887	0,887	0,887	0,887	0,993	0,982	1,000
BRsw09pdm-JQ666846_A/swine/Brazil/2/2009_2009/08/11	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,988	0,967	0,973	0,970	0,970	0,891	0,889	0,887	0,887	0,887	0,887	0,993	0,982	1,000	1,000
BRsw09pdm-JQ666847_A/swine/Brazil/3/2009_2009/08/11	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,988	0,967	0,973	0,970	0,970	0,891	0,889	0,887	0,887	0,887	0,887	0,993	0,982	1,000	1,000	1,000
BRsw09pdm-JQ666848_A/swine/Brazil/4/2009_2009/08/21	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,963	0,969	0,967	0,967	0,887	0,885	0,882	0,883	0,883	0,883	0,990	0,979	0,997	0,997	0,997	1,000
BRsw09pdm-JQ666849_A/swine/Brazil/5/2009_2009/08/28	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,982	0,959	0,966	0,964	0,964	0,891	0,889	0,887	0,887	0,887	0,887	0,986	0,982	0,994	0,994	0,994	0,991	1,000
BRsw09pdm-JQ666850_A/swine/Brazil/6/2009_2009/08/28	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,982	0,959	0,966	0,964	0,964	0,891	0,889	0,887	0,887	0,887	0,887	0,986	0,982	0,994	0,994	0,994	0,991	1,000	1,000
BRsw09pdm-JQ666851_A/swine/Brazil/7/2009_2009/09/26	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,988	0,967	0,973	0,970	0,970	0,891	0,889	0,887	0,887	0,887	0,887	0,993	0,982	1,000	1,000	1,000	0,997	0,994	0,994	1,000
BRsw09pdm-JQ666852_A/swine/Brazil/8/2009_2009/10/05	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,963	0,970	0,967	0,967	0,888	0,885	0,883	0,884	0,884	0,884	0,990	0,979	0,997	0,997	0,997	0,994	0,991	0,991	0,997	1,000
BRsw09pdm-JQ666853_A/swine/Brazil/9/2009_2009/10/05	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,963	0,970	0,967	0,967	0,888	0,885	0,883	0,884	0,884	0,884	0,990	0,979	0,997	0,997	0,997	0,994	0,991	0,991	0,997	1,000	1,000
BRsw09pdm-JQ666854_A/swine/Brazil/10/2009_2009/10/05	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,982	0,959	0,966	0,964	0,964	0,884	0,881	0,879	0,880	0,880	0,880	0,986	0,976	0,994	0,994	0,994	0,991	0,988	0,988	0,994	0,997	0,997	1,000
BRsw09pdm-JQ666855_A/swine/Brazil/11/2009_2009/10/18	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,988	0,967	0,973	0,970	0,970	0,891	0,889	0,887	0,887	0,887	0,887	0,993	0,982	1,000	1,000	1,000	0,997	0,994	0,994	1,000	0,997	0,997	0,994	1,000
BRsw09pdm-JQ666856_A/swine/Brazil/12/2009_2009/10/25	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,963	0,970	0,967	0,967	0,894	0,893	0,890	0,891	0,891	0,891	0,990	0,985	0,997	0,997	0,997	0,994	0,997	0,997	0,997	0,994	0,994	0,991	0,997	1,000
BRsw09pdm-JQ666857_A/swine/Brazil/13/2009_2009/11/10	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,963	0,970	0,967	0,967	0,894	0,893	0,890	0,891	0,891	0,891	0,990	0,985	0,997	0,997	0,997	0,994	0,997	0,997	0,997	0,994	0,994	0,991	0,997	1,000	1,000
BRsw09pdm-JQ666858_A/swine/Brazil/14/2009_2009/12/04	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,963	0,969	0,967	0,967	0,895	0,893	0,891	0,891	0,891	0,891	0,990	0,979	0,997	0,997	0,997	0,994	0,991	0,991	0,997	0,994	0,994	0,991	0,997	0,994	0,994	1,000
BRsw09pdm-JQ666859_A/swine/Brazil/15/2009_2009/12/21	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,988	0,967	0,973	0,970	0,970	0,891	0,889	0,887	0,887	0,887	0,887	0,993	0,982	1,000	1,000	1,000	0,997	0,994	0,994	1,000	0,997	0,997	0,994	1,000	0,997	0,997	0,997	1,000
BRsw09pdm-JQ666861_A/swine/Brazil/17/2009_2009/08/01	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,988	0,967	0,973	0,970	0,970	0,891	0,889	0,887	0,887	0,887	0,887	0,993	0,982	1,000	1,000	1,000	0,997	0,994	0,994	1,000	0,997	0,997	0,994	1,000	0,997	0,997	0,997	1,000	1,000
BRsw09pdm-JQ666860_A/swine/Brazil/16/2009_2009/08/01	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,988	0,967	0,973	0,970	0,970	0,891	0,889	0,887	0,887	0,887	0,887	0,993	0,982	1,000	1,000	1,000	0,997	0,994	0,994	1,000	0,997	0,997	0,994	1,000	0,997	0,997	0,997	1,000	1,000	1,000
BRsw09pdm-KM497008_A/swine/Brazil/132/2009_2009/09/09	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,991	0,988	0,967	0,973	0,970	0,970	0,891	0,889	0,887	0,887	0,887	0,887	0,993	0,982	1,000	1,000	1,000	0,997	0,994	0,994	1,000	0,997	0,997	0,994	1,000	0,997	0,997	0,997	1,000	1,000	1,000	1,000
BRsw10pdm-JF421756_A/swine/Brazil/12A/2010_2010/02/02	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,985	0,982	0,959	0,966	0,964	0,964	0,883	0,881	0,878	0,879	0,879	0,879	0,986	0,976	0,994	0,994	0,994	0,991	0,988	0,988	0,994	0,991	0,991	0,988	0,994	0,991	0,991	0,991	0,994	0,994	0,994	0,994	1,000
BRsw10pdm-JQ666862_A/swine/Brazil/18/2010_2010/05/11	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,963	0,970	0,967	0,967	0,888	0,885	0,883	0,884	0,884	0,884	0,990	0,979	0,997	0,997	0,997	0,994	0,991	0,991	0,997	0,994	0,994	0,991	0,997	0,994	0,994	0,994	0,997	0,997	0,997	0,997	0,991	1,000
BRsw10pdm-JQ666863_A/swine/Brazil/19/2010_2010/09/09	0,982	0,982	0,982	0,982	0,982	0,982	0,982	0,982	0,982	0,982	0,979	0,963	0,963	0,961	0,961	0,881	0,881	0,883	0,877	0,877	0,877	0,990	0,973	0,991	0,991	0,991	0,988	0,985	0,985	0,991	0,988	0,988	0,985	0,991	0,988	0,988	0,988	0,991	0,991	0,991	0,991	0,985	0,988	1,000
BRsw10pdm-JQ666864_A/swine/Brazil/20/2010_2010/09/09_	0,982	0,982	0,982	0,982	0,982	0,982	0,982	0,982	0,982	0,982	0,979	0,963	0,963	0,961	0,961	0,881	0,881	0,883	0,877	0,877	0,877	0,990	0,973	0,991	0,991	0,991	0,988	0,985	0,985	0,991	0,988	0,988	0,985	0,991	0,988	0,988	0,988	0,991	0,991	0,991	0,991	0,985	0,988	1,000	1,000
BRsw10pdm-KF683614_A/swine/Brazil/107-3A/2010_2010/07/14	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,967	0,969	0,967	0,967	0,887	0,889	0,887	0,883	0,883	0,883	0,993	0,979	0,997	0,997	0,997	0,994	0,991	0,991	0,997	0,994	0,994	0,991	0,997	0,994	0,994	0,994	0,997	0,997	0,997	0,997	0,991	0,994	0,988	0,989	1,000
2017/18/19-KY117023.1_(A/Michigan/45/2015(H1N1))	0,976	0,976	0,976	0,976	0,976	0,976	0,976	0,976	0,976	0,976	0,973	0,955	0,963	0,961	0,961	0,894	0,893	0,890	0,891	0,891	0,891	0,976	0,979	0,985	0,985	0,985	0,982	0,985	0,985	0,985	0,982	0,982	0,979	0,985	0,988	0,988	0,982	0,985	0,985	0,985	0,985	0,979	0,982	0,976	0,972	0,978	1,000
2010/16-KU933485.1_Influenza_A_virus_(A/California/07/2009(H1N1))	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,963	0,970	0,967	0,967	0,887	0,885	0,883	0,884	0,884	0,884	0,990	0,979	0,997	0,997	0,997	0,994	0,991	0,991	0,997	0,994	0,994	0,991	0,997	0,994	0,994	0,994	0,997	0,997	0,997	0,997	0,991	0,994	0,988	0,986	0,992	0,975	1,000
H1N1npdm_A-CA-4-2009	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,988	0,985	0,963	0,970	0,967	0,967	0,887	0,885	0,883	0,884	0,884	0,884	0,990	0,979	0,997	0,997	0,997	0,994	0,991	0,991	0,997	0,994	0,994	0,991	0,997	0,994	0,994	0,994	0,997	0,997	0,997	0,997	0,991	0,994	0,988	0,986	0,992	0,975	1,000	1,000

A

B

C

Partial sequences of H1N1pdm09 were aligned using CluskalW method on Bioedit Program. Number of base differences per site from between sequences were calculated using p-distance method on Mega7 software. Sequences in light gray refer to Group A and are from 2014/2015. Sequences in silver refer to group B and are from 2012/2013, Sequences in gray refer to group C and are from 2014/2015. Dark gray sequences are from 2012/2013. Sample sequence demarcated by black border refers to the licensed vaccine strain for swine in Brazil.

Supplementary Table 7. P-distance analysis of partial sequences of H1Delta subtype

	BRsw14/15Delta-Z2D	BRsw14/15Delta-Z3D	BRsw12/13Delta-FLU13D	BRsw14/15Delta-Z47D	BRsw14/15Delta-_Z24D	BRsw17/18Delta_-JE1D	BRsw17/18Delta-_552D	BRsw17/18Delta-IP1006D	BRsw17/18Delta-553D	BRsw12/13Delta-_FLU4D	BRsw17/18Delta_-_JH1D	BRsw17/18Delta-_P1D	BRsw12/13Delta-_FLU11D	2009-JN899402.1_Influenza_A_virus_(A/Brisbane/59/2007(H1N1))	KM507530.1(A/swine/Brazil/232-11-13/2011(H1N2))	KM507532.1(A/swine/Brazil/232-11-14/2011(H1N2)	KP027585.1(A/swine/Brazil/G2P1/2013(H1N2))	KM507519.1(A/swine/Brazil/185-11-7/2011(H1N2))	KF680296.1_I(A/swine/Brazil/31_11_1/2011(H1N2))	KF680291.1_I(A/swine/Brazil/31_11_3/2011(H1N2))	KF572616.1_I(A/wild_boar/Brazil/214-11-13D/2011(H1N2))	MG572188.1_(A/swine/Brazil/103-2/2014(H1N1))	KP458442.1_I(A/New_York/310/2001(H1N1))	AB117205.1_I(A/Miyagi/4/2000(H1N1))
BRsw14/15Delta-Z2D	1,000																							
BRsw14/15Delta-Z3D	0,987	1,000																						
BRsw12/13Delta-FLU13D	1,000	0,987	1,000																					
BRsw14/15Delta-Z47D	1,000	0,987	0,995	1,000																				
BRsw14/15Delta-_Z24D	0,993	0,992	0,985	0,985	1,000																			
BRsw17/18Delta_-JE1D	0,897	0,877	0,893	0,898	0,887	1,000																		
BRsw17/18Delta-_552D	0,874	0,852	0,875	0,879	0,860	0,995	1,000																	
BRsw17/18Delta-IP1006D	0,919	0,917	0,919	0,919	0,910	0,889	0,860	1,000																
BRsw17/18Delta-553D	0,893	0,880	0,899	0,892	0,899	0,917	0,899	0,961	1,000															
BRsw12/13Delta-_FLU4D	0,912	0,897	0,918	0,910	0,911	0,930	0,908	0,957	0,974	1,000														
BRsw17/18Delta_-_JH1D	0,921	0,903	0,906	0,918	0,924	0,944	0,925	0,961	0,968	1,000	1,000													
BRsw17/18Delta-_P1D	0,921	0,903	0,906	0,918	0,924	0,944	0,927	0,961	0,969	1,000	1,000	1,000												
BRsw12/13Delta-_FLU11D	0,800	0,789	0,800	0,800	0,828	0,783	0,750	0,809	0,780	0,812	0,802	0,802	1,000											
2009-JN899402.1_Influenza_A_virus_(A/Brisbane/59/2007(H1N1))	0,955	0,946	0,949	0,943	0,947	0,928	0,915	0,910	0,897	0,917	0,919	0,925	0,820	1,000										
KM507530.1(A/swine/Brazil/232-11-13/2011(H1N2))	0,981	0,967	0,965	0,974	0,970	0,906	0,884	0,928	0,912	0,925	0,917	0,922	0,769	0,942	1,000									
KM507532.1(A/swine/Brazil/232-11-14/2011(H1N2)	1,000	1,000	0,929	0,978	1,000	0,875	0,861	0,952	0,926	0,899	0,899	0,899	0,545	0,972	0,999	1,000								
KP027585.1(A/swine/Brazil/G2P1/2013(H1N2))	0,968	0,953	0,955	0,963	0,963	0,886	0,873	0,919	0,901	0,895	0,894	0,903	0,778	0,930	0,982	0,981	1,000							
KM507519.1(A/swine/Brazil/185-11-7/2011(H1N2))	0,988	0,974	0,971	0,979	0,978	0,902	0,878	0,919	0,906	0,918	0,913	0,918	0,779	0,939	0,994	0,995	0,980	1,000						
KF680296.1_I(A/swine/Brazil/31_11_1/2011(H1N2))	0,928	0,910	0,912	0,923	0,931	0,955	0,943	0,918	0,930	0,939	0,962	0,955	0,787	0,920	0,944	0,942	0,933	0,944	1,000					
KF680291.1_I(A/swine/Brazil/31_11_3/2011(H1N2))	0,928	0,910	0,912	0,923	0,931	0,955	0,943	0,918	0,930	0,939	0,962	0,955	0,787	0,923	0,945	0,942	0,932	0,945	0,999	1,000				
KF572616.1_I(A/wild_boar/Brazil/214-11-13D/2011(H1N2))	0,928	0,910	0,912	0,923	0,931	0,955	0,943	0,918	0,930	0,939	0,962	0,955	0,787	0,923	0,944	0,942	0,932	0,944	0,999	0,999	1,000			
MG572188.1_(A/swine/Brazil/103-2/2014(H1N1))	0,907	0,888	0,906	0,912	0,915	0,940	0,932	0,910	0,930	0,960	0,958	0,952	0,781	0,927	0,920	0,916	0,908	0,920	0,920	0,922	0,921	1,000		
KP458442.1_I(A/New_York/310/2001(H1N1))	0,955	0,939	0,934	0,946	0,955	0,951	0,942	0,944	0,941	0,953	0,967	0,966	0,820	0,963	0,951	0,945	0,943	0,950	0,958	0,958	0,958	0,952	1,000	
AB117205.1_I(A/Miyagi/4/2000(H1N1))	0,962	0,946	0,944	0,952	0,963	0,955	0,942	0,936	0,936	0,947	0,963	0,963	0,829	0,970	0,951	0,941	0,940	0,952	0,956	0,958	0,958	0,951	0,991	1,000

A

B

B.1

B.2

Partial sequences of H1Delta were aligned using CluskalW method on Bioedit Program. Number of base differences per site from between sequences were calculated using p-distance method on Mega7 software. In mid gray there are sequences from group A, mostly from 2013/2014. Sequences in light gray refer to Group B which is subdivided in B.1 and B.2. Sequences from group B are mostly from 2017/2018. Sequence in dark gray refers to sample Flu11D. In white there are sequences previously deposited in Genbank.

Supplementary Table 8. P-distance analysis of partial sequences of H3N2 subtype

	BRsw17/18H3N2-_JE2	BRsw17/18H3N2-_553H3	BRsw17/18H3N2-_552H3	BRsw14/15H3N2-_Z31H3	BRsw17/18H3N2-_TRAQ1201	BRsw12/13H3N2_-_FLu3H3	BRsw17/18H3N2_-_JHH3	BRsw17/18H3N2_-Cat3	BRsw17/18H3N2_-_SarH3	DQ336017.1_I(A/Brazil/013/00(H3N2))	KT781412.1_I(A/Sao_Paulo/34/1995(H3N2))	DQ336016.1_I(A/Brazil/011/00(H3N2))	AF311677.1_I(A/Finland/380/95(H3N2))	AF008734.1_I(A/Brazil/2/95(H3N2))	EF456786.1_I(A/England/255/1995(H3N2))	AF180665.1_I(A/Johannesburg/53/96(H3N2))	CY011808.1_I(A/New_York/647/1995(H3N2))	AY661188.1_I(A/Victoria/75/95(H3N2))	CY012216.1_I(A/New_York/639/1995(H3N2))	CY112765.1_I(A/Hong_Kong/32/1995(H3N2))	AF534022.1_I(A/Buenos_Aires/4634/96(H3N2))	KM507511.1(A/swine/Brazil/365-11-7/2011(H3N2))	KM507503.1(A/swine/Brazil/355-11-6/2011(H3N2))	KM507535.2(A/swine/Brazil/231-11-1/2011(H3N2))	EF456791.1_I(A/England/286/1995(H3N2))	MH814933.1_I(A/swine/Minnesota/A01785592/2018(H3N2))	MK071676.1_I(A/swine/Missouri/A02257614/2018(H3N2))_	MG870262.1_I(A/swine/Iowa/A02139351/2018(H3N2))	MK156668.1_I(A/swine/Alabama/A02267936/2018(H3N2))	MH345756.1_I(A/swine/Tennessee/A02157798/2018(H3N2))	AF153233.1_I(A/Swine/Texas/4199-2/98_(H3N2))	CY095675.1_I(A/swine/Texas/4199-2/1998(H3N2))	AF153235.1_I(A/Swine/Iowa/8548-1/98)	AF251427.2_I(A/Swine/Minnesota/593/99_(H3N2))	AF251403.1_I(A/Swine/Nebraska/209/98_(H3N2))	AF251419.1_I(A/Swine/Iowa/569/99_(H3N2))
BRsw17/18H3N2-_JE2	1,000																																				
BRsw17/18H3N2-_553H3	1,000	1,000																																			
BRsw17/18H3N2-_552H3	1,000	1,000	1,000																																		
BRsw14/15H3N2-_Z31H3	1,000	1,000	1,000	1,000																																	
BRsw17/18H3N2-_TRAQ1201	0,996	0,995	0,995	0,994	1,000																																
BRsw12/13H3N2_-_FLu3H3	1,000	1,000	1,000	1,000	0,995	1,000																															
BRsw17/18H3N2_-_JHH3	1,000	1,000	1,000	1,000	0,995	1,000	1,000																														
BRsw17/18H3N2_-Cat3	1,000	1,000	1,000	1,000	0,994	1,000	1,000	1,000																													
BRsw17/18H3N2_-_SarH3	0,929	0,916	0,915	0,911	0,920	0,916	0,926	0,911	1,000																												
DQ336017.1_I(A/Brazil/013/00(H3N2))	0,980	0,989	0,985	0,988	0,977	0,989	0,986	0,988	0,929	1,000																											
KT781412.1_I(A/Sao_Paulo/34/1995(H3N2))	0,972	0,984	0,980	0,982	0,973	0,984	0,978	0,982	0,922	0,990	1,000																										
DQ336016.1_I(A/Brazil/011/00(H3N2))	0,980	0,989	0,985	0,988	0,977	0,989	0,986	0,988	0,929	0,998	0,992	1,000																									
AF311677.1_I(A/Finland/380/95(H3N2))	0,976	0,989	0,985	0,988	0,977	0,989	0,982	0,988	0,927	0,998	0,992	1,000	1,000																								
AF008734.1_I(A/Brazil/2/95(H3N2))	0,976	0,989	0,985	0,988	0,977	0,989	0,982	0,988	0,928	0,998	0,992	1,000	0,999	1,000																							
EF456786.1_I(A/England/255/1995(H3N2))	0,976	0,989	0,985	0,988	0,977	0,989	0,982	0,988	0,928	0,998	0,992	1,000	0,999	0,998	1,000																						
AF180665.1_I(A/Johannesburg/53/96(H3N2))	0,967	0,979	0,975	0,976	0,968	0,979	0,973	0,976	0,922	0,982	0,975	0,984	0,989	0,988	0,988	1,000																					
CY011808.1_I(A/New_York/647/1995(H3N2))	0,976	0,989	0,985	0,988	0,977	0,989	0,982	0,988	0,931	0,996	0,991	0,998	0,998	0,997	0,997	0,989	1,000																				
AY661188.1_I(A/Victoria/75/95(H3N2))	0,976	0,989	0,985	0,988	0,977	0,989	0,982	0,988	0,925	0,996	0,991	0,998	0,999	0,998	0,998	0,988	0,996	1,000																			
CY012216.1_I(A/New_York/639/1995(H3N2))	0,976	0,989	0,985	0,988	0,977	0,989	0,982	0,988	0,928	0,995	0,989	0,996	0,997	0,996	0,996	0,986	0,993	0,994	1,000																		
CY112765.1_I(A/Hong_Kong/32/1995(H3N2))	0,963	0,979	0,975	0,976	0,968	0,979	0,968	0,976	0,930	0,991	0,985	0,993	0,995	0,994	0,994	0,985	0,989	0,994	0,988	1,000																	
AF534022.1_I(A/Buenos_Aires/4634/96(H3N2))	0,963	0,979	0,975	0,976	0,968	0,979	0,969	0,976	0,927	0,987	0,981	0,989	0,991	0,990	0,990	0,979	0,989	0,990	0,988	0,994	1,000																
KM507511.1(A/swine/Brazil/365-11-7/2011(H3N2))	0,924	0,916	0,916	0,905	0,915	0,917	0,926	0,905	0,959	0,915	0,909	0,917	0,921	0,920	0,923	0,914	0,932	0,920	0,932	0,932	0,918	1,000															
KM507503.1(A/swine/Brazil/355-11-6/2011(H3N2))	0,929	0,922	0,921	0,911	0,920	0,923	0,931	0,912	0,963	0,912	0,906	0,915	0,923	0,923	0,924	0,912	0,926	0,924	0,924	0,927	0,918	0,980	1,000														
KM507535.2(A/swine/Brazil/231-11-1/2011(H3N2))	0,933	0,922	0,921	0,911	0,925	0,922	0,930	0,911	0,963	0,921	0,918	0,923	0,923	0,922	0,925	0,913	0,931	0,923	0,931	0,932	0,918	0,977	0,975	1,000													
EF456791.1_I(A/England/286/1995(H3N2))	0,976	0,989	0,985	0,988	0,977	0,989	0,982	0,988	0,928	0,996	0,991	0,998	0,998	0,997	0,997	0,987	0,996	0,997	0,995	0,993	0,989	0,924	0,925	0,926	1,000												
MH814933.1_I(A/swine/Minnesota/A01785592/2018(H3N2))	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,920	0,968	0,967	0,970	0,971	0,970	0,970	0,959	0,975	0,974	0,974	0,970	0,963	0,920	0,908	0,918	0,971	1,000											
MK071676.1_I(A/swine/Missouri/A02257614/2018(H3N2))_	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,922	0,968	0,967	0,970	0,973	0,972	0,972	0,961	0,975	0,974	0,974	0,970	0,965	0,919	0,908	0,916	0,973	0,995	1,000										
MG870262.1_I(A/swine/Iowa/A02139351/2018(H3N2))	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,920	0,968	0,967	0,970	0,973	0,972	0,972	0,961	0,977	0,976	0,975	0,972	0,965	0,921	0,909	0,919	0,973	0,997	0,997	1,000									
MK156668.1_I(A/swine/Alabama/A02267936/2018(H3N2))	0,980	0,995	0,995	1,000	0,977	0,995	0,991	0,994	0,920	0,965	0,965	0,967	0,969	0,968	0,968	0,957	0,974	0,972	0,972	0,969	0,959	0,918	0,906	0,916	0,969	0,993	0,993	0,995	1,000								
MH345756.1_I(A/swine/Tennessee/A02157798/2018(H3N2))	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,922	0,966	0,965	0,968	0,971	0,970	0,970	0,959	0,976	0,974	0,975	0,971	0,963	0,921	0,909	0,919	0,971	0,996	0,996	0,998	0,994	1,000							
AF153233.1_I(A/Swine/Texas/4199-2/98_(H3N2))	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,918	0,968	0,967	0,970	0,974	0,973	0,973	0,962	0,975	0,977	0,973	0,973	0,967	0,909	0,907	0,910	0,974	0,997	0,998	0,999	0,994	0,998	1,000						
CY095675.1_I(A/swine/Texas/4199-2/1998(H3N2))	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,918	0,968	0,967	0,970	0,974	0,973	0,973	0,962	0,978	0,977	0,977	0,973	0,967	0,922	0,908	0,921	0,974	0,997	0,997	0,999	0,995	0,998	1,000	1,000					
AF153235.1_I(A/Swine/Iowa/8548-1/98)	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,920	0,967	0,963	0,968	0,971	0,970	0,970	0,959	0,973	0,972	0,971	0,970	0,963	0,911	0,909	0,915	0,971	0,989	0,990	0,991	0,985	0,989	0,992	0,992	1,000				
AF251427.2_I(A/Swine/Minnesota/593/99_(H3N2))	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,920	0,968	0,965	0,970	0,973	0,972	0,972	0,961	0,977	0,974	0,975	0,973	0,965	0,922	0,912	0,922	0,973	0,991	0,991	0,993	0,989	0,992	0,994	0,994	0,997	1,000			
AF251403.1_I(A/Swine/Nebraska/209/98_(H3N2))	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,920	0,968	0,965	0,970	0,973	0,972	0,972	0,961	0,975	0,973	0,974	0,972	0,965	0,921	0,912	0,922	0,973	0,991	0,991	0,992	0,988	0,992	0,993	0,993	0,996	0,999	1,000		
AF251419.1_I(A/Swine/Iowa/569/99_(H3N2))	0,984	1,000	1,000	1,000	0,982	1,000	0,996	1,000	0,920	0,968	0,965	0,970	0,973	0,972	0,972	0,961	0,975	0,973	0,974	0,971	0,965	0,920	0,911	0,920	0,973	0,990	0,990	0,992	0,988	0,991	0,992	0,992	0,995	0,999	0,999	1,000	

A

Partial sequences of H3N2 were aligned using CluskalW method on Bioedit Program. Number of base differences per site from between sequences were calculated using p-distance method on Mega7 software. Sequences in light gray refer to Group A. Sequence in dark gray refers to the sample SarH3D. In white are sequences previously deposited in Genbank.