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Swine influenza A virus subtypes circulating in Brazilian commercial pig herds from 2012 to 2019

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Abstract

The swine influenza A virus (SIAV) subtypes/lineages H1N1pdm09, H3N2, H1N2, and H1N1 of seasonal human origin are widespread in Brazilian swine herds. A monovalent inactivated H1N1pdm09 vaccine was licensed in Brazil in 2014. However, there are concerns about its efficacy due to the limited vaccine cross-protection against heterologous viruses and the potential for exacerbated reactions against vaccine strains. Thus, monitoring SIAVs subtypes/lineages that are circulating in the Brazilian swine population is important, by applying a fast and efficient diagnostic test in herd field samples. A RT-PCR assay was developed, using primers specific for HA subtyping of Brazilian SIAV, and was used to evaluate the occurrence of subtypes from samples collected between 2012 and 2019. From 167 field samples positive for influenza A, 117 were subtyped by nested RT-PCR assay. A higher occurrence of H1N1pdm was observed from 2012 to 2015, H3N2 in 2017, and H1hu in 2017 to 2019. A hemagglutination inhibition test was performed in serum samples received from 2017 to 2019, confirming these data. The molecular data highlights the importance of H1hu and H3N2 detection since there are no vaccines available for the subtypes/lineages and raises an alert of H1hu for its potential to infect humans. Serological data suggest a cyclical profile of occurrence between the H3N2 and H1N1pdm over time. Monitoring SIAVs circulating in Brazilian swine herds is necessary, which provides the relevant information for field veterinarians to apply effective control measures on the properties.

Keywords Influenza virus · Brazil · Genotyping · H1N1pdm · H1hu · H3N2 · Serology · Swine

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Introduction

Among the respiratory diseases that affect swine production, infection with swine influenza A virus (SIAV) is one of the most striking since it may cause explosive outbreaks of acute respiratory disease, affecting almost 100% of the herd, and its clinical signs can be exacerbated by coinfections with bacteria and other viruses [1]. The disease leads to a reduction in feed intake and weight loss, causing substantial economic losses to the pork industry. Moreover, swine influenza is a major concern to humans due to the risk that these viruses can cross the species barrier and be transmitted to humans [2, 3].

The subtypes of SIAV that infects pigs worldwide belong to the species *Influenza A virus*, genus *Alphainfluenzavirus*, family *Orthomyxoviridae*, and its genome is composed of eight negative-polarity single-stranded segments that encode at least

10 viral proteins. Due to the segmented genotype and the lack of proofreading by the RNA polymerase during virus replication, reassortments and mutations are common, contributing to influenza A virus (IAV) variability. The hemagglutinin (HA) and neuraminidase (NA) glycoproteins are the main targets of the host immune response and are highly subject to mutations, contributing to virus evolution and evasion of the host immune response [4].

Worldwide, different subtypes of SIAV circulate in the swine population: H1N1, H1N2, H3N2. In Brazil, SIAV has been a major threat to the pork industry since the emergence of the 2009 pandemic influenza virus in humans, when several outbreaks associated with the H1N1pdm09 virus were described in swine farms [5]. Since then, this virus seems to have become endemic in Brazilian pig herds, and its emergence favors reassortments with other circulating SIAVs, producing multiple new strains and increasing its genetic diversity [3]. Currently, the subtypes/lineages that are present and widespread in Brazil include H3N2, H1N2, and H1N1 seasonal human origin and the 2009 H1N1 pandemic virus [6, 7].

In 2014, a monovalent inactivated vaccine encoding the hemagglutinin protein of the 2009 pandemic H1N1 lineage was licensed in Brazil [8], and so far, it is the only one commercially vaccine available for use in Brazil, in addition to autogenous vaccines that are also allowed for use only to the target herd or adjacent properties [9]. Although there are no studies about the coverage of vaccine use in Brazilian herds, there are concerns about the use of the monovalent commercial vaccine, whether it provides sufficient protection to herds since studies have indicated that the vaccine elicited poor cross-protection against heterologous viruses [8, 9] and there are different strains circulating in herds, reducing the usefulness of vaccination for Brazilian herds.

Because of the challenges involving viral genetic variability, such as coinfection, multiple strains circulating in pigs, and poor vaccine cross-protection, the monitoring of viral subtypes/lineages circulating in the swine population is very relevant to animal health. The application of rapid and efficient diagnostic tests in affected herds allows for the monitoring of viral subtypes in the country and provides the necessary information, allowing veterinarians to undertake efficient control measures on farms. Efficient control measures by vaccination can only be performed by knowing the HA subtypes/lineages that are circulating in the swineherds, such as H1pdm09, H1hu (seasonal human origin), and H3. Moreover, since the majority of available swine influenza vaccines are inactivated, including the only one commercially available in Brazil, knowing the circulating SIAVs in Brazilian herds is essential. This information is important, as vaccine protection is due mainly to humoral immune response and it is deeply impacted by antigenic mismatches among circulating strains and vaccine formulations.

The aim of this work was to evaluate the occurrence of different subtypes/lineages of the swine influenza virus circulating in Brazilian herds, by the analysis of clinical samples collected between 2012 and 2019, using a nested RT-PCR assay developed and validated in this study. Serological analysis was also performed in serum samples collected from 2017 to 2019.

Material and methods

Nested RT-PCR for SIAV detection of HA gene subtypes/lineages

Primer design

To increase the sensitivity of the nested RT-PCR assay for detection of different SIAV subtypes and lineages for Brazilian field samples, primers were designed for the partial detection of the HA gene in the H1pdm09, H3, and H1hu sequences. The sequences of the HA glycoprotein gene from Brazilian swine influenza viruses were selected from the GenBank Database (Supplementary Table 1) and aligned by ClustalW multiple sequence alignment [10]. The primers were designed using Primer-BLAST software. The sequences were aligned separately by HA subtype (H1 or H3), and conserved regions of H1 and H3 were used to design the external primers. Using the selected regions, internal conserved regions of H1pdm, H1hu, and H3 were chosen to design the primers for nested RT-PCR. Information about the primers used for PCRs is shown in Table 1, as well as the primer positions, amplified base pair fragments, and respective melting temperatures.

Reference viruses

The H1N1pdm09, H3N2, and H1N1hu viruses used as positive controls for nested RT-PCR assays were previously characterized by RT-PCR and sequencing [11, 12] (Table 2). Following the guidelines of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [13], the strains were isolated by sequenced passages in *Madin-Darby canine kidney* (MDCK) cell culture from lung fragments of animals that presented respiratory clinical signs on Brazilian pig farms.

Development of the nested RT-PCR assay

Viral RNA was extracted using TRIzol® reagent (Thermo Fisher, USA) according to the manufacturer's instructions. RNA was stored at − 80°C.

For the first reaction, the viral genome was reverse transcribed and amplified using a Superscript One-Step RT-PCR System with Platinum Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA). All samples were subjected to a standardized PCR containing 1× PCR buffer, 0.4 μM of each primer, 200 μM of each dNTP, varying concentrations of $MgSO_4$ (1.5 mM and 2 mM), 1.5 U of Taq DNA polymerase, and 2.5 μL of the extracted RNA, for a final volume of 25 μL. The

Table 1 Sets of primers sequences used in nested RT-PCR assays

Reaction subtype/lineage	Set of primers	Primers sequences (5'–3')	Position	Amplicon length (bp)	Tm (°C)
1st reaction/H1	FW1H1	AGCAAAAGCAGGGGAAAAYA	781–798	616bp	50
	RV1H1	CCCCATAGYAYRAGGACTTC	534–553		
2nd reaction/H1pdm	FW2H1pdm	CCAATCATGACTCGAACAAC	391–410	159bp	52
	RV2H1pdm	ATAGCACGAGGACTTCTTTCT	529–549		
2nd reaction/H1hu	FW2H1hu	GCGTTGCCGGATGGATCTTA	233–252	303bp	55
	RV2H1hu	ACAGACCATTCTTCCCCGTCA	515–535		
1st reaction/H3	FW1H3	ATATGTTCAAACGTCAGGGAGA	651–672	544bp	52
	RV1H3	GTCGATTGCTGCTTGAGTGC	1175–1194		
2nd reaction/H3	FW1H3	ATATGTTCAAACGTCAGGGAGA	651–672	279bp	52
	RV2H3	TTCCCATTTGGAGTGACGCA	910–929		

cycling conditions included an initial reverse transcription at 48 °C or 50 °C for 30 min, followed by a denaturation step at 95 °C for 2 min and 40 cycles of denaturation at 95 °C for 30 s, an annealing step varying from 50 to 57 °C, and an extension step at 64 °C for 1 min, followed by a final extension step at 68 °C for 5 min.

In the second reaction, 1 μL of an amplicon was amplified in a 25 μL reaction containing 1× PCR buffer, 0.4 μM of each primer, varying concentrations of $MgSO_4$ (1.5 mM and 2 mM), 200 μM of each dNTP, 1 U of Taq DNA polymerase (Phoneutria Biotecnologia, Brazil), and ultrapure nuclease-free water to the final volume.

The nested reaction started with a denaturation temperature of 95 °C for 5 min followed by 40 cycles of a denaturation step at 95 °C for 30 s, an annealing step varying from 52 to 57°C for 30 s, and an extension step at 68 °C for 1 min, followed by a final extension step at 68 °C for 5 min. The PCR amplicons were identified by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

Sensitivity of the nested RT-PCR assay

The method of limiting dilution was used to evaluate the analytic sensitivity of the nested and one-step RT-PCR assays. The primer sets were tested for the detection of the three reference swine influenza viruses. The H1N1pdm09 and H3N2 isolates, with titers of 4×10^5 plaque-forming units (PFU)/mL, were serially diluted 10-fold (10^{-1} up to 10^{-14}), and RNA was extracted from 300 μL of each dilution. It was not possible to titrate H1N1hu isolate because its RNA was already extracted. From the extracted RNA, a 10-fold serial dilution was performed to be used in the RT-PCR assay [14, 15].

Specificity of the nested RT-PCR assay

To test the specificity of the nested RT-PCR assay, all the primer sets from the second amplification reaction were tested for each reference sample. Cross-reactivity with DNA viruses and bacteria that infect swine, such as porcine circovirus type 2 and *Mycoplasma hyopneumoniae* were evaluated as well as for RNA virus such as Senecavirus A and rotavirus A, B, and C.

Evaluation of nested RT-PCR assay using clinical samples

Samples received for the diagnosis of SIAV 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), were tested for the influenza A matrix gene by RT-PCR [5]. A total of 167 field samples (146 lung tissues and 21 nasal swabs), positive for the influenza A matrix gene, were then analyzed for SIAV differentiation by nested RT-PCR. These samples were collected from animals presenting respiratory clinical signs from non-vaccinated commercial pig

Table 2 The H1N1pdm09, H1N1hu, and H3N2 virus strains were used as positive controls for HA nested RT-PCR assays

Strain	State origin/country	Species	Collection year	Reference strain (HA gene)
H1N1pdm09	Minas Gerais/Brazil	Swine	2009	JQ666849*
H1N1hu	Santa Catarina/Brazil	Swine	2013	EPI_ISL_575027**
H3N2	Paraná/Brazil	Swine	2017	EPI_ISL_574871**

*GenBank accession number

**GISAID accession number

herds mostly located in states with the highest pork production in Brazil: Santa Catarina (SC), Paraná (PR), Rio Grande do Sul (RS), Minas Gerais (MG), São Paulo (SP), and Mato Grosso do Sul (MS) and Goiás (GO) (Supplementary Table 2) in the years 2012–2013, 2014–2015, 2017–2018, and 2019. As these samples were received for a diagnosis, much information related to the age of the sampled animals and the exact location of the herd's properties were not sent by the field veterinarians that requested the exams. These data were then missed, which made it impossible for us to trace more details of the received samples, with the exception of the year of collection, city, and state of origin.

RT-PCR for NA gene subtyping

For NA gene (N1 and N2) subtyping, specific primers and PCR protocols were used according to Choi et al. [14].

Sequencing

The resulted RT-PCR amplicons from the first reaction of positive H1pdm samples and from the second reaction of H3 and H1hu positive samples were purified using a DNA Clean and Concentrator kit (Zymo Research, USA) and subsequently dosed by a NanoDrop spectrophotometer (Thermo Fisher, USA). Samples with more than 40 ng were selected for sequencing by the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher, USA). Sequencing reactions were performed by capillary electrophoresis on an ABI 3730 instrument (Applied Biosystems, USA). The nucleotide sequence data were analyzed and assembled using SeqMan Software 7.1.0 (DNAStar Inc.). Consensus sequences were compared with sequences reported in GenBank (Supplementary Table 1), and those with an identity percentage higher than 95% using BLAST (Basic Local Alignment Search Tool) from the NCBI (National Center for Biotechnology) were selected.

Phylogenetic analyses of the HA gene fragments of the Brazilian influenza virus samples were performed by Bayesian inference. For each influenza subtype/lineage H1pdm, H1hu, and H3, newly generated sequences were aligned with references obtained from the GISAID and GenBank databases (Supplementary Table 4 using MAFFT v7.471 [16] with global pair, adjustment direction, and 1000 iterations. The alignment was manually polished in AliViewV1.2.6 [17] (Larsson, 2014) to remove gaps that were present in less than 10% of the isolates and overhangs, resulting in final alignment sequences of 319, 195, and 156 nucleotides for H1pdm09, H1hu, and H3, respectively. The phylogeny was estimated using MrBayesV3.2.7a [18], with 3 million MCMC replicates, 25% burnin, and the HKY + I nucleotide substitution model for H3N2 and GTR + I for H1N1 and H1hu, which was selected using JmodelTestV2.1.10 based on the AIC

approach [19] (Posada, 2008). The tree image was generated with the ITOL online tool [20] (Letunic and Bork, 2019).

Serology

Between 2017 and 2019, a total of 1631 serum samples from non-vaccinated pig farms located in different Brazilian states were received at the LPVA for diagnosis (Supplementary Table 3). In this period, 263 samples were obtained from 2017, 677 from 2018, and 691 from 2019. A hemagglutination test for detection of the viral titers in hemagglutinating units (UHA) was performed using the H1N1pdm09 and H3N2 reference viruses (Table 2) according to the protocol described by WHO (2002) [21]. Due to the low hemagglutination titer of the H1N1hu isolated reference strain, it was not possible to use it in serological analyzes. Samples with an HI titer ≥ 40 were considered positive.

Results

Optimization of nested RT-PCR assays

The concentration of $MgCl_2$ for the one-step RT-PCR and second round of PCR was 2 mM, which minimized the occurrence of nonspecific bands. The annealing temperatures that minimized the occurrence of nonspecific 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 H1hu, respectively) for nested RT-PCR. Therefore, the cycling conditions for the first RT-PCR were an initial reverse transcription at 48 °C or 50 °C (for H1 and H3, respectively) for 30 min, followed by a denaturation step at 95 °C for 2 min and then 40 cycles of denaturation at 95 °C for 30 s, an annealing step at 50 °C or 52 °C (for H1 and H3, respectively) for 30 s, and an extension at 64 °C for 1 min, followed by a final extension step at 68 °C for 5 min. The second round of PCR started with a denaturation temperature of 95 °C for 5 min followed by 40 cycles of a denaturation step at 95 °C for 30 s, annealing at 52 °C, 52 °C, or 55 °C (for H1N1pdm09, H3N2, and H1hu, respectively) for 30 s, and extension at 68 °C for 1 min, followed by a final extension step at 68 °C for 5 min.

Sensitivity and specificity of nested RT-PCR assays

To assess the sensitivity of nested RT-PCR, a 15-fold dilution for H1N1pdm09 and H3N2 and a 10-fold dilution for H1N1hu were performed. The sensitivity results of the assay are shown in Supplementary Figure 1. The limit of detection of the nested RT-PCR for H1N1pdm09 was 10^{-11}, a 10^8 higher than that of the one-step RT-PCR, which used the first set of primers. For H3N2, the limit of detection was 10^{-9},

which was different from the first reaction, that was only 10^{-5}. The detection limit for H1N1hu was lower, corresponding to 10^{-4}, near the limit of the first reaction, which was 10^{-3}.

To determine the specificity of the assay, the three SIAV reference samples (H1N1pdm09, H1N1hu, and H3N2) were tested with all three sets of primers used in the nested RT-PCR. All three subtypes/lineages yielded the expected base pair fragments, which were 159 bp, 303 bp, and 279 bp for H1pdm09, H1hu, and H3, respectively. It should be noted that when the H1N1pdm09 sample was tested with the H1hu primers, a band near 600 bp was detected representing amplification of the fragment related to the first RT-PCR (616 bp for H1), but there was no amplification of the 303-bp band, which is specific for H1hu (Supplementary Figure 2). When cross-tested against nonspecific primers for each viral subtype/lineage, none of the samples yielded any amplification product (Supplementary Figure 2). Cross-reactivity with other swine RNA and DNA swine pathogens was not observed confirming the specificity of the test

In addition, to confirm the specificity of the nested RT-PCR, the amplicons were sequenced and analyzed by molecular phylogeny. The partial genome sequence of field samples indicated the specific subtype/lineage in accordance with the nested RT-PCR results.

Evaluation of field sampling

Of the 167 positive samples for the influenza A matrix gene, the detection of the HA subtype/lineage was done in 117 (117/167–70%) by nested RT-PCR. Fifty-five samples were positive for H1N1pdm (55/117–47%), 29 for H1hu (29/117–24.8%), and 10 for H3N2 (10/117–8.5%) (Table 3).

Mixed infections with two subtypes/lineages (H1N1pdm + H1hu; H1hu + H3N2; H1N1pdm09 + H3N2) were 10/117–8.5%, 6/117–5.1%, and 4/117–3.4%, respectively, and those with all subtypes/lineages (H1N1pdm + H1hu + H3N2) were 3/117–2.6%.

Analyzing the 29 H1hu-positive samples, eight samples were subtyped for the NA gene. Results have shown five samples (5/29–17.2) positive for N1 and three (3/29–10.3%) for N2. In 21/29–72.4% of the H1hu-positive samples, NA gene subtyping was not successful, probably due to the coinfection of these samples with H1pdm (10/29–34.5%), H3N2 (6/29–20.7%), or both subtypes/lineages (3/29–10.3%).

Analysis of 117 subtyped samples showed that 14 (14/117–12%) were from 2012–2013; 35 (35/117–30%) were from 2014–2015; 22 (22/117–19%) were from 2017–2018; and 46 (46/117–39%) were from 2019. Among the samples from 2012–2013, 64.3% were positive for H1N1pdm and 21.4% for H1hu. Coinfections between H1N1pdm and H3N2 were observed in 14.3% of the samples. In this period, H3N2 single infections and coinfections were not observed among the three subtypes/lineages. In 2014–2015, the results

showed that 77.1% of the samples were positive for H1N1pdm, and only 2.9% and 11.4% were positive for H3N2 and H1hu, respectively. In these years, coinfections were observed only between H1N1pdm and H1hu, corresponding to 8.6% of the samples.

Analyzing the occurrence of different SIAVs in 2017–2018, the percentage of samples positive for H1N1pdm was 22.7%, which was lower than that in the other years. However, an increase in H3N2-positive samples to 22.7% (5/22) was observed, equaling the percentage obtained for H1N1pdm. Samples positive for H1hu were found at a higher percentage than H1N1pdm and H3N2, corresponding to 27.2%. Coinfections between the three subtypes/lineages were detected in 13.6% of the samples. In addition, coinfections between the pairs H1N1pdm + H3N2, H1N1pdm + H1hu, and H3N2 + H1hu were observed at 4.6% for each combination. In 2019, the occurrence of H1hu was the highest (34.8%), followed by H1N1pdm (30.4%). Coinfections between H1N1pdm + H1hu also increased, with an occurrence of 13%. A significant decrease in H3N2 in single infections (8.7%) and coinfections of H3N2 + H1N1pdm (2.2%) occurred, although infections with H1hu increased (10.9%).

The relative occurrence of H1N1pdm, H3N2, and H1hu by year, considering the total number of performed subtyping analyses and not considering coinfections, is shown in Figure 1. In 2012–2013, the most detected lineage was H1N1pdm (68.8%–11/16), followed by H1hu (18.8%–3/16) and H3N2 (12.5%–2/16). In 2014–2015, H1N1pdm remained the most detected (78.9%–30/38), followed by H1hu (18.4%–7/38) and H3N2 (2.6%–1/38).

However, in 2017–2018, there was a balance among the assessed influenza subtypes/lineages. Indeed, an increase in the occurrences of the H3N2 and H1hu was observed, with the H3N2 and H1N1pdm showing equivalence of 32.3% (10/31) and H1hu demonstrating 35.5% (11/31), the highest occurrence among the analyzed samples. Increased occurrences of H1N1pdm and H1hu were also observed in 2019 compared to 2017–2018, with 36.2% (21/58) and 46.6% (27/58) in the subtypes sampled, respectively. In 2019, the occurrence of H1hu remained higher than that of H1N1pdm09, while the detection of H3N2 was 17.2% (10/58).

Serology

Of the 1631 serum samples analyzed by the HI test, 1220 (75%) were positive for IAV. In the sera collected in 2017, HI analysis detected the highest occurrence of H1N1pdm + H3N2 coinfections, 42.6% (112/263), followed by specific antibodies for single infections of H3N2 and H1N1pdm in 32.7% (86/263) and 10.6% (28/263) of serum samples, respectively. In 2018, serum samples that were only positive for H3N2 (39.9%–270/677) were also more prevalent than

Table 3 Total subtyped samples in 2012–2013, 2014–2015, 2017–2018, and 2019

Subtypes/lineages	Sampling date (year)			
	2012/2013	2014/2015	2017/2018	2019
H1N1pdm	9/14 (64.3%)	27/35 (77.1%)	5/22 (22.7%)	14/46 (30.4%)
H1hu	3/14 (21.4%)	4/35 (11.4%)	6/22 (27.2%)	16/46 (34.8%)
H1N1hu	1/3 (33.3%)	4/4 (100%)	0/6 (0%)	0/46 (0%)
H1N2hu	2/3 (66.6%)	0/4 (0%)	1/6 (16%)	0/16 (0%)
H3N2	0/14 (0%)	1/35 (2.9%)	5/22 (22.7%)	4/46 (8.7%)
H1N1pdm + H1hu	0/14 (0%)	3/35 (8.6%)	1/22 (4.6%)	6/46 (13%)
H1N1pdm + H3N2	2/14 (14.3%)	0/35 (0%)	1/22 (4.6%)	1/46 (2.2%)
H3N2 + H1hu	0/14 (0%)	0/35 (0%)	1/22 (4.6%)	5/46 (10.9%)
All mixed subtypes/lineages	0/14 (0%)	0/35 (0%)	3/22 (13.6%)	0/46 (0%)
Total subtyped samples	14	35	22	46

those that were only positive for H1N1pdm (3%–23/677). A large occurrence of H1N1pdm + H3N2 (27.8%–188/677) was observed at that time. In 2019, in sharp contrast to our previous finding in 2017 and 2018, we detected the highest occurrence of specific antibodies for H1N1pdm (43.8%–303/691), followed by coinfections at 27.1% (187/691) and a low occurrence of specific antibodies to H3N2 (3.3%–23/691) (Figure 2).

Serological data from received samples suggest that there is a cyclical profile of occurrence between the H3N2 and H1N1pdm in Brazilian swine herds each year and that H1N1pdm is not always the most prevalent subtype/lineage.

Sequencing and phylogenetic analysis of the partial HA gene

Thirty-nine amplicons were sequenced: twenty-two amplicons from H1pdm, ten from H3, and seven from H1hu. The nucleotide sequences of the HA nucleotide sequences were deposited

in the GISAID database, and information regarding the subtype/lineage, state, year of collection, and GISAID accession numbers is listed in Supplementary Table 5. As different genetic regions were amplified between subtypes/lineages, phylogenetic analyses were performed separately. The use of short sequences could reduce phylogenetic reliability and that the use of larger sequences, as the complete HA region, could be more informative. However, there are still some relevant differences in the evaluated fragments to allow potential phylogenetic estimations by robust methods (Supplementary Figure 3). Hence, we used Bayesian inference, one of the best methods to estimate phylogeny, to try to obtain some reliable phylogenetic signal that could be lost in other simpler approaches, as Neighbor Joining and Maximum Parsimony. For this reason, there were several branches that were strongly supported in the threes, with close to 100 posterior probability values. These branches could be at least considered, even with the short sequences. The sequence variability and phylogenetic analysis of the HA partial gene can be seen in Supplementary Figures 4, 5 and 6.

Fig. 1 Percentage of samples positive for H1N1pdm, H3N2, and H1hu in relation to the total of samples determined by subtyping analyses performed in 2012–2019, not considering coinfections
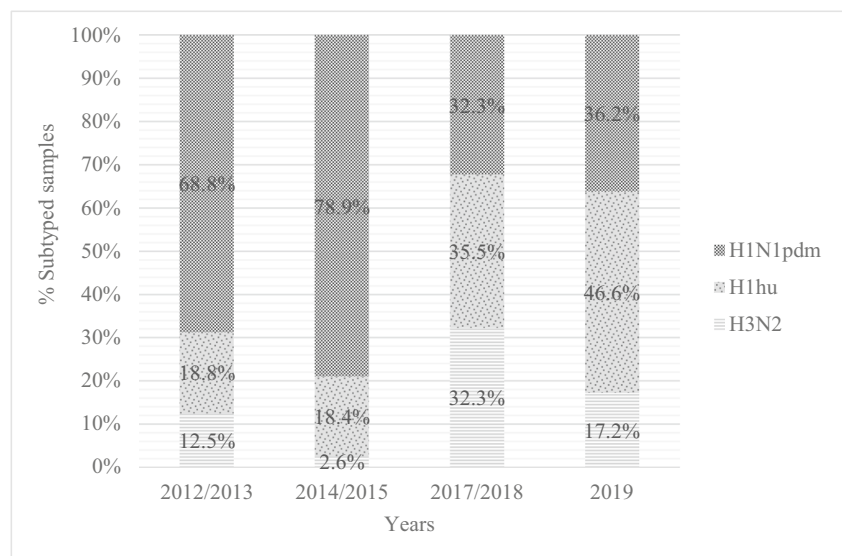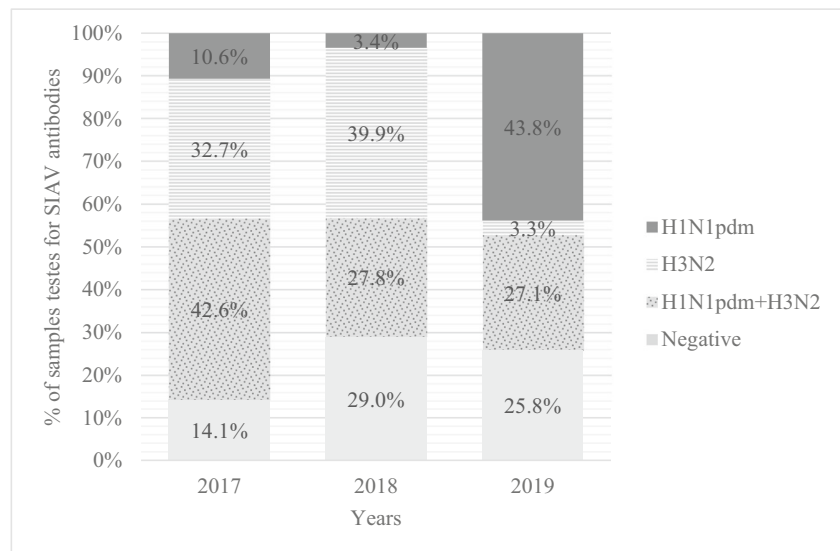
Fig. 2 Percentage ratio of samples tested for antibodies against H1N1pdm, H3N2, and H1N1pdm + H3N2 in 2017, 2018, and 2019

Discussion

Monitoring circulating SIAV subtypes/lineages is necessary and essential for epidemiological surveillance, helping veterinarians to establish prevention and treatment measures in affected farms, as well as for vaccine development. Rapid, sensitive, and specific diagnostic tests are important to monitor circulating viruses. In Brazil, no commercial tests are available to subtype SIAV. Some studies used next-generation sequencing (NGS) techniques to obtain a complete sequence of the viral genome from field samples [5, 7], which is very efficient in obtaining genetic information but is not a feasible large-scale strategy since it is laborious, time-consuming, and expensive. Some studies using multiplex real-time RT-PCR (RT-qPCR) for the detection of H3 and different H1 lineages circulating in Europe, subtyped between 89 and 94% of SIAV-positive clinical samples [15, 22, 23]. Another study using a one-step multiplex RT-qPCR assay for the identification of different subtypes and lineages of SIAV circulating in Brazil demonstrated high sensitivity and specificity when applied for the diagnostic of IAV in clinical samples [24]. However, real-time PCR requires a greater investment and qualified technicians, which could be limitations for diagnostic labs and customers. High sensitivity in subtyping multiplex RT-PCR was demonstrated mostly when applied for SIAV isolates [25, 26]. Other studies have developed multiplex RT-PCR tests using primer sets that distinguish H1 and H3 but do not cover the differentiation of H1 lineages [14, 27–29]. In our study, we highlighted a high-sensitivity subtyping diagnostic technique in clinical samples. Using the developed nested RT-PCR and applying it to clinical samples, it was possible to release results in less than 24 h to field veterinarians, making viral isolation to increase the viral load for posterior subtyping, which takes 2–3 weeks, unnecessary. Moreover, the developed test was standardized using primers specifically delineated from HA Brazilian swine influenza virus sequences, increasing its specificity and importance of use to subtype field samples for monitoring SIAV in Brazil. Furthermore, and with greater importance, using the test to analyze clinical samples collected in 2012–2013, 2014–2015, and 2017–2019, it was possible to detect the four SIAV subtypes/lineages: H1N1pdm09, H1hu (H1N1 and H1N2), and H3N2.

Remarkably, our technique enabled us to demonstrate by nested PCR that between 2012–2013 and 2014–2015, a higher detection of H1N1pdm was observed, followed by H1hu, mixed infections between H1hu + H1N1pdm and H3N2 + H1N1pdm, and H3N2. The subtyping data corroborate with previously published serological studies, which have shown that H1N1pdm is endemic in Brazil. A study performed in 2011 in the RS, PR, SC, SP, MG, and MS states showed that the H1N1pdm lineage was more prevalent in animal herds, followed by H1N2 and H3N2, and a high prevalence of coinfections between the lineages with the involvement of H1N1pdm was also observed [7]. In 2012, another study performed in swine farms in the state of MG indicated that 26.2% of the tested animals had antibodies against H1N1pdm and only 1,57% had antibodies against H3N2 [6]. In 2014–2015, a serological study performed by our group in unvaccinated farms from several Brazilian states showed that 96% of the herds were positive for H1N1pdm and 68% were positive for H3N2, of which 36% presented coinfections with H1N1pdm [30]. With the serological studies already published, the PCR subtyping data corroborate the higher occurrence of H1N1pdm in the 2012–2015 period. Coinfections between different SIAVs were also observed, calling attention to the higher probability of the occurrence of reassortments between different subtypes/lineages. The higher detection of H1hu, especially in the PCR subtyping study, reinforces that this human origin lineage circulates in Brazilian swine herds and seems to have settled in this population [3, 31, 32].

Compared to previous years, in 2017–2018, serological and PCR subtyping data from clinical samples revealed a significant increase in the occurrence of H3N2, as well as in H1N1pdm + H3N2, and a decrease in H1N1pdm. The results of molecular analysis corroborate with data previously published demonstrating a higher detection of H3N2 in clinical samples in this period [24]. The increasing occurrence of H3N2 in swine may be related to the occurrence of this IAV subtype in the human population. According to human serological data published by the WHO (2018) [33] in 2017, a greater and significant occurrence of H3N2 was observed in the Brazilian human population, while the occurrence of H1N1pdm was not expressive, mostly because of the introduction of a new H1N1pdm vaccine strain into the human vaccine formulation. Data from swine and human IAV show a parallel occurrence of the virus subtypes in both species and monitoring IAV circulating in the human population is a tool that may help forecast possible outbreaks of influenza in swine. The increase of H3N2 occurrence in pigs in 2018, evidenced mainly by serological data of samples collected from non-vaccinated farms, may be related to the lower contact of pigs with H1N1pdm since this lineage circulated with a low prevalence in the human population. It is worth mentioning that transmission of influenza from humans to pigs, mainly of the H1N1pdm, has been shown to be more frequent and constant than the reverse route [34], and biosafety measures are important to prevent this transmission.

In contrast, in 2019, the H1hu lineage was the most prevalent when considering single inflections or coinfections in nested PCR analysis, followed by H1N1pdm, which had the highest incidence in serology. This result suggests a cyclic profile of the influenza virus and the importance of human seasonal subtype/lineage that are constantly circulating in swine herds. There are no H1hu and H3N2 vaccines available in Brazil. Vaccination of swineherds, with H1N1pdm09 vaccines, which are constantly challenged with H1hu and H3N2, in addition to not offering efficient cross-protection, may lead to vaccine-associated enhanced respiratory disease (VAERD) [31], which may occur when vaccinated pigs are challenged with heterologous viruses. Piglets vaccinated with delta-1 cluster H1N2 (H1hu) and challenged with H1N1pdm09 virus have demonstrated enhanced clinical signs of the disease and higher lung lesion scores than non-vaccinated/challenged animals [35]. Furthermore, the high occurrence of H1hu in the swine population raises an alert for its potential to cause massive infection in humans that are susceptible to the lineage. To date, in Brazil, only occasional cases of H1N1hu and H1N2hu have been reported [31, 32, 36, 37], and those have been found in people who worked and had contact with sick pigs. Nonetheless, our data show that the circulation of influenza viruses of variable strains and subtypes/lineages poses an unneglectable risk both to pig farmers and the general population. Therefore, strict SIAV surveillance and the development of safer, more efficient vaccines should be developed to solve this problem.

This study had some limitations related to the sampling. As all samples of the study were received in our laboratory for the diagnosis of respiratory diseases, we could not determine the prevalence of SIAV subtypes/lineages in Brazilian herds, but we were able to estimate their occurrence by demonstrating a profile of which subtypes/lineages were circulating in the herds in the analyzed period. Due to missed data about the samples, we were unable to analyze the occurrence of the strains by age or category as well as their frequency of occurrence in the herds, in both serological and molecular analyses. The sequencing of the subtyped samples by the RT-PCR was able to confirm the specificity of the standardized molecular test; however, we could not robustly discuss the phylogenetic analysis due to the small size of the sequences

In summary, it was possible to subtype and differentiate SIAVs that are circulating in Brazilian swine herds by the RT-PCR developed in the present study, without the need for viral isolation, demonstrating the importance of monitoring viruses in the field samples and contributing to epidemiological surveillance. The data presented in this study suggest that the occurrence of different subtypes/lineages, demonstrated by both RNA detection and serology, varies over time within an annual cyclical period. Surveillance of influenza virus in humans and pigs using genetic characterization is an important auxiliary tool to prevent possible influenza outbreaks in both species and to establish updates for vaccines. To obtain more consistent data and accurate analyses, further studies analyzing the complete HA gene of SIAVs circulating in Brazil from different years must be performed.

Author contributions • Ana Luiza Soares Fraiha and Ana Carolina Diniz Matos: Sample processing, acquisition of data, analysis and interpretation, drafting the article

• João Luis Reis Cunha: construction of phylogenetic data and analysis, drafting the article • Maria Vitória Peixoto Chaves, Grazielle Cossenzo Florentino Galinari, Anna Gabriella Guimarães Oliveira and Heloiza Irtes de Jesus Nascimento: Sample processing and acquisition of data

• Ana Carolina Diniz Matos, Beatriz Senra Álvares da Silva Santos, Alexandre de Magalhães Vieira Machado, Maria Isabel Maldonado Coelho Guedes: Critical review of the article

• Zélia Inês Portela Lobato and Erica Azevedo Costa: Conception and design of the study, drafting, and review of the article

• Zélia Inês Portela Lobato: Final approval of the version to be submitted

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Declarations

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Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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