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VETERINARY AND ANIMAL PRODUCTION - RESEARCH PAPER

Passive surveillance for Influenza A virus among swine, Brazil, 2009–2023

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Abstract

Influenza A virus (IAV) is present in most swine-producing countries causing production losses and concerns on public health. In Brazil, influenza is endemic in pig herds, and a great genetic diversity has been described in swine IAVs due to multiple introductions of pre-2009 human-seasonal IAVs followed by reassortment events with 2009 pandemic H1N1 (H1N1pdm) virus. Here, we compile 14 years of IAV monitoring data and describe the subtypes and major lineages of H1 and H3 viruses co-circulating in Brazilian pigs. Using multiplex RT-qPCR and sequencing, we identified H1N1pdm as the most frequently detected virus, accounting for 41.3% of the subtyped samples (165/399), followed by H1huN2 (108/399), H3N2 (77/399), and H1N1hu (9/399). The three dominant subtypes were detected co-circulating annually and consistently in seven of the nine states sampled, as well as among pigs at different production phases. Other reassortants were found sporadically and included H1pdmN2 (22/399) and H1huN1pdm (4/399). The high diversity observed indicates that IAVs from distinct lineages are widely disseminated across the country. These findings strongly suggest substantial movement of pigs between regions and states, which may have implications for vaccine design, disease control, and updating of diagnostic tests. Continuous efforts to monitor IAV are crucial to better understand their ecology and to generate relevant data for pandemic preparedness.

Keywords Influenza · Genetic diversity · Respiratory infection · RT-qPCR · Subtyping · Swine

Introduction

Influenza A virus (IAV) is present in most swine-producing countries causing production losses and concerns on public health. The virus belongs to the family *Orthomyxoviridae* and is classified within the genus *Alphainfluenzavirus* [1]. The single-stranded segmented RNA genome of IAV facilitates genetic reassortment and contributes to the emergence of new viral strains. Differences in the antigenic and genetic properties of the two major glycoproteins on the viral envelope - hemagglutinin (HA) and neuraminidase (NA) - are used to define IAV subtypes [2]. To date, 18 HAs and 11 NAs have been detected in several host species; however, only three subtypes - H1N1, H1N2, and H3N2 - predominate in swine globally [2, 3]. Influenza viruses exhibit a high mutation rate and a remarkable capacity for genetic diversification, which facilitates quick adaptation to new hosts. The rapid evolution is driven by two mechanisms: point mutations and reassortment [4]. Accumulation of point mutations can alter their antigenic properties, allowing the viruses to evade the host immune system. Reassortment occurs when two different IAVs co-infect the same host cell and exchange gene segments, leading to the emergence of novel viral constellations and, occasionally, strains with whole new antigenic profiles. Ultimately, interspecies transmission between humans and swine has profoundly shaped the evolutionary history of IAV in pigs, and multiple lineages of swine IAVs (swIAV) circulate globally [5].

Respiratory infections in pigs, known as porcine respiratory disease complex (PRDC), are a major concern for swine producers, leading to increased medication costs, higher mortality rates, and reduced animal performance. IAV is

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one of the most relevant pathogens in PRDC [6] and, alone or in co-infection with *Mycoplasma hyopneumoniae*, is recognized as the main pathogen involved in PRDC in Brazil [7–9]. Before 2009, there were few reports of influenza and serologic studies revealed circulation of classical H1N1 and H3N2 viruses [10, 11]. After 2009, outbreaks of influenza infection became frequent in pig herds coinciding with the emergence of the 2009 pandemic H1N1 virus (H1N1pdm) in humans [12, 13]. Currently, influenza is enzootic in Brazilian pig herds [7, 8, 14], and a vast genetic diversity has been observed among swIAVs of the H1N1, H1N2, and H3N2 subtypes circulating in the country. These viruses are genetically and antigenically distinct from those found in swine in other countries, mainly due to incursions of human seasonal viruses at different time points, followed by reassortment events among enzootic IAVs and the H1N1pdm virus [3, 15–18].

The HA of swIAVs can be classified according to a global nomenclature system that designates three major lineages of H1 viruses (1A, 1B, and 1C), and several lineages of H3 viruses based on the decade (1970, 1990, 2000 and 2010) of circulation of their closest related human-origin ancestral [5, 19]. H1 viruses of 1A lineage are derived from the 1918 Spanish flu H1N1 virus. It was transmitted to pigs and evolved into the classical H1N1 swine lineage that spread globally (North America, Asia, Europe). The 1A lineage also includes the H1 HA of the H1N1pdm virus, which after spreading in humans, was repeatedly reintroduced into swine populations globally, and formed the genetic clade 1A.3.3.2 [3]. The 1B lineage resulted from multiple spillover events of pre-2009 human seasonal H1 viruses over several decades, while the 1C lineage emerged following the introduction of an avian IAV into pigs in the 1970s [5]. Differently from H1, H3 lineages are geographically distinct, except for clade 1990.4, which was detected in the U.S, Canada, Mexico, and Korea [19].

Based on this classification, viruses of two H1 lineages (1A and 1B) and one H3 lineage (from the 1990s) have been identified in Brazil [15], and at least six phylogenetic clades appear to co-circulate in the Brazilian swine population [16, 17]. Distinct human-to-swine transmission events involving pre-2009 H1N1 and H1N2 human seasonal viruses resulted in three genetic clades of Brazilian swIAVs within 1B lineage. These viruses carry a human-like H1 (H1hu), derived from strains that circulated in the mid-to-late 1980s and early 2000s - clades 1B.2.3, 1B.2.4, and 1B.2.6 [16]. Since 2009, multiple human-to-swine spillover events have introduced the 2009 pandemic H1 (H1pdm) into Brazilian pigs, resulting in at least four sustained transmission clades [17]. These viruses belong to clade 1A.3.3.2 - the only 1A clade with widespread global distribution, reported in 12 countries [15, 17]. Brazilian swine H3 viruses originated from a seasonal H3N2 human virus introduced into pigs in the late 1990s. This single introduction gave rise to the H3 1990.5 lineage, which diversified into three genetic clades: 1990.5.1, 1990.5.2, and 1990.5.3 [16]. Additionally, phylogenetic analysis of the NA gene revealed further introductions of human-origin IAVs over time, resulting in the presence of three distinct NA genetic clades in Brazilian swine: the 2009 pandemic N1 (N1pdm), a human-like N1 (N1hu), and an N2 [16, 17].

Although the zoonotic and pandemic potential of swIAV has been repeatedly recognized [20], there are currently no sustained government-administered surveillance programs in Brazil. Usually, a veterinary practitioner collects samples from pigs suspected of having an influenza infection on farms and submits the selected biological samples to a diagnostic laboratory. IAV positive samples are submitted for molecular testing and/or virus isolation in research laboratories. Embrapa Swine and Poultry works closely with the Ministry of Agriculture and Livestock (MAPA) to monitor diseases in pig herds in Brazil. Passive monitoring of IAV in pigs has been conducted since 2009, when histological lesions compatible with IAV infection were observed in pigs showing respiratory clinical signs, leading to the identification of the pandemic H1N1 influenza virus [12]. For the diagnosis of influenza, sampling should be directed to pigs in the acute phase of the disease (showing fever, cough, labored abdominal breathing) to increase the chances of virus detection. The techniques of choice for detecting IAV are RT-PCR and virus isolation (VI) from both nasal swabs and lung tissue samples, or detection of the viral antigen associated with lesions of bronchointerstitial pneumonia in lungs by IHC [21]. Although these techniques are widely used in diagnostic laboratories, they do not identify the virus subtype or genetic lineage, besides being laborious. This information is highly relevant for the selection of representative virus strains for vaccine production.

In this study, we compiled and provided a detailed description of data obtained by Embrapa using a multiplex RT-qPCR [22] and sequencing of the HA and NA, describing the frequency and distribution of IAV subtypes and major lineages circulating in pigs from commercial herds from the main pig producing regions of the country.

Materials and methods

Pig sampling

From August 2009 to January 2023, 743 biological samples (nasal swab or lung tissue) were collected from pigs exhibiting respiratory clinical signs in 602 commercial herds and sent to two private veterinary diagnostic laboratories for

respiratory pathogens screening. The biological samples were obtained from pigs at different ages and production phases: suckling piglets (1–3 weeks old), weaning (3–9 weeks old), growing/finishing (9–21 weeks old), and breeding pigs (gilts, sows, and boars), and raised in nine Brazilian states from South (Paraná, Rio Grande do Sul, and Santa Catarina), Southeast (Minas Gerais and São Paulo), and Midwest (Distrito Federal, Goiás, Mato Grosso, and Mato Grosso do Sul) regions. These selected Brazilian states are the country's leading pork producers. Pig farming in these three regions is characterized by modern, technified systems and includes integrated, cooperative, and independent producers. Production is commonly organized across separate facilities, with different farms dedicated to specific stages of the production cycle - farrowing, nursery, and finishing. During sampling, animals were observed for clinical signs suggestive of influenza infection, such as fever, labored abdominal breathing, and coughing.

IAV detection and viral isolation

Initially, samples were screened by real-time RT-PCR in two private veterinary diagnostic laboratories. IAV- positive samples were then sent to the Brazilian Agricultural Research Corporation (EMBRAPA Swine and Poultry) for virus isolation (VI), subtyping, and sequencing. Briefly, viral RNA was extracted with the MagMAX™ Viral RNA Isolation Kit (Ambion), and complementary DNA (cDNA) synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturers' protocols. Screening of IAV positive samples was performed using a real-time RT-PCR assay designed to target the M gene [23]. Positive samples were submitted to VI in SPF-embryonated chicken eggs or MDCK cells [24]. Inoculated eggs were incubated at 37 °C for 3–4 days, after which the allantoic fluid was harvested and tested for viral presence. Alternatively, MDCK cells were cultured at 37 °C with 5% CO_2 and monitored for cytopathic effects (CPE) up to 5–7 days, indicative of viral replication. VI was confirmed by real time RT-PCR [23] after up to two viral passages. Viral isolates were kept at -70 °C until further analysis.

Swine IAV subtyping

One or two influenza virus isolates per herd were selected to determine the hemagglutinin (HA) and neuraminidase (NA) subtypes. For this, viral RNA was extracted using the RNeasy® mini kit (Qiagen, Germany) according to the manufacturer's recommendations. The RT-qPCR assay is a multiplex TaqMan-based method, consisting of six different primers and probes designed to detect the HA and NA subtypes and lineages from Brazilian swine IAVs. Reactions were carried out using the AgPath-ID One-Step RT-PCR Kit (4387391, Ambion) [22]. The IAV subtype and lineage were determined using one of the following approaches: (a) by an one-step multiplex RT-qPCR assay that distinguishes the three major lineages of HA (H1pdm or 1A.3.3.2, H1 human-like or 1B.2, and H3) and NA genes (N1pdm, N1 human-like, and N2) circulating in swine in Brazil [22] or (b) by whole-genome next-generation sequencing using the Ion Torrent system (Thermo Fisher Scientific®, Waltham, MA, USA). Initially, multiplex RT-qPCR was used to evaluate samples with a cycle threshold (Ct) value lower than 28, as determined by screening real-time RT-PCR (targeting the M gene) [23]. If the test provided partial subtypes, sequencing was used to define the viral subtype.

For DNA sequencing, the eight influenza gene segments were amplified by RT-PCR using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase following the manufacturer's guidelines (PCR amplification of influenza A genomic segments for whole-genome sequencing, Ion Torrent sequencing application guide; Thermo Fisher Scientific®, Waltham, MA, USA). Influenza genomes were assembled using Newbler v.2.9 (Roche, USA). The major lineage of HA and NA segments was confirmed with the aid of *octoFLU* classifier pipeline [25] followed by phylogenetic inference including all the Brazilian sequences available up to the time this study was written [16, 17].

Results

Subtypes of IAV detected in Brazilian pigs from 2009 to 2023

In this study, we used a multiplex RT-qPCR for detection, subtyping and for classification of H1N1, H1N2 and H3N2 viruses according to their genetic lineages. To facilitate interpretation, we established the following nomenclature based on hemagglutinin (HA) and neuraminidase (NA) origins: H1N1pdm: HA and NA derived from pandemic 2009 H1N1 virus; H1N1hu: HA and NA derived from pre-2009 human seasonal H1N1 influenza virus; H1huN2: HA and NA derived from pre-2009 human seasonal H1N2 influenza virus; H3N2: HA and NA derived from human seasonal H3N2 influenza virus; H1huN1pdm: HA derived from pre-2009 human seasonal H1N1 influenza virus and NA derived from pandemic 2009 H1N1 virus; H1pdmN2: HA derived from pandemic 2009 H1N1 virus and N2 derived from human seasonal H1N2 or H3N2 influenza virus.

Between 2009 and 2023, a total of 399 influenza A viruses were isolated and subtyped by multiplex RT-qPCR. Overall,

viruses from the lineage H1N1pdm were the most frequently detected (152/399), followed by H1huN2 (107/399), H3N2 (74/399), and H1N1hu (9/399). Reassortant viruses were found in 26 of the samples and included H1pdmN2 (22/399) and H1huN1pdm (4/399). Mixed infections (i.e., when two HA/NA subtypes are detected in the same sample) were identified in six of the samples and included H1pdm + H3/N2 (n = 2), H1pdm/N1pdm + N2 (n = 2), H1pdm + H1hu/N2 (n = 1), H1hu + H3/N2 (n = 1). In 25 of the samples only the HA or the NA could be subtyped. Of these 25 samples partially subtyped by multiplex RT-qPCR, sequencing defined the viral subtype for 17 samples, resulting in 13 H1N1pdm, three H3N2, and one H1huN2. Eight samples remained partially subtyped after sequencing: four N1pdm, two N2, and two H3. After sequencing resolved partial subtypes, the final frequencies of each virus subtype were: H1N1pdm (41.3%), H1huN2 (27%), H3N2 (19.3%), H1N1hu (2.3%), H1pdmN2 (5.5%), and H1huN1pdm (1%), with 2% of samples remaining partially subtyped (Fig. 1a). The distribution of subtypes detected annually is shown in Fig. 1b.

Subtypes of IAV according to the state of origin

IAV samples were obtained from nine Brazilian states located in three regions: South (Rio Grande do Sul, Santa Catarina, and Paraná), Southeast (Minas Gerais and São Paulo), and Midwest (Mato Grosso, Mato Grosso do Sul, Goiás, and Distrito Federal) (Fig. 2a). These samples were distributed across 602 commercial herds (Fig. 2b). The sampled states together account for over 70% of the national swine population [26]. The swine herd size per state, the number of sampled herds per state, and the number of virus lineages detected in each Brazilian state are shown in Fig. 2a and b, and Table 1.

Subtypes of IAV by production phase

Most of the samples analyzed here were obtained from weaning pigs (58%; 231/399), followed by growing/finishing (19.3%; 77/399), suckling piglets (5.3%; 21/399), and breeding swine (2.8%; 11/399). The age of the animals was unknown in 11.3% (45/399) of the cases (ni, not informed) (Fig. 3). The three dominant subtypes (H1N1pdm, H1huN2, and H3N2) were detected in all pig categories (Fig. 3). In addition, mixed infections were detected in five weaning pigs from different municipalities, and in one suckling piglet. Partial subtyping was obtained for samples collected from weaning pigs (5/231), and growing/finishing (2/77), and suckling piglets (1/21).

Discussion

In this study, we described the subtypes and main lineages of H1 and H3 influenza A virus circulating in swine during 2009–2023 using either a multiplex RT-qPCR and sequencing. Virus strains were collected from farms located in nine Brazilian states, from three regions, that concentrate most of the intensive pig production and together represent 99.87% of pork production in Brazil [27].

During 2009 and 2010, the number of subtyped samples was lower (n = 19/399), most of the samples were from the South region of Brazil and H1N1pdm was the virus lineage most frequently detected. This finding agrees with previous

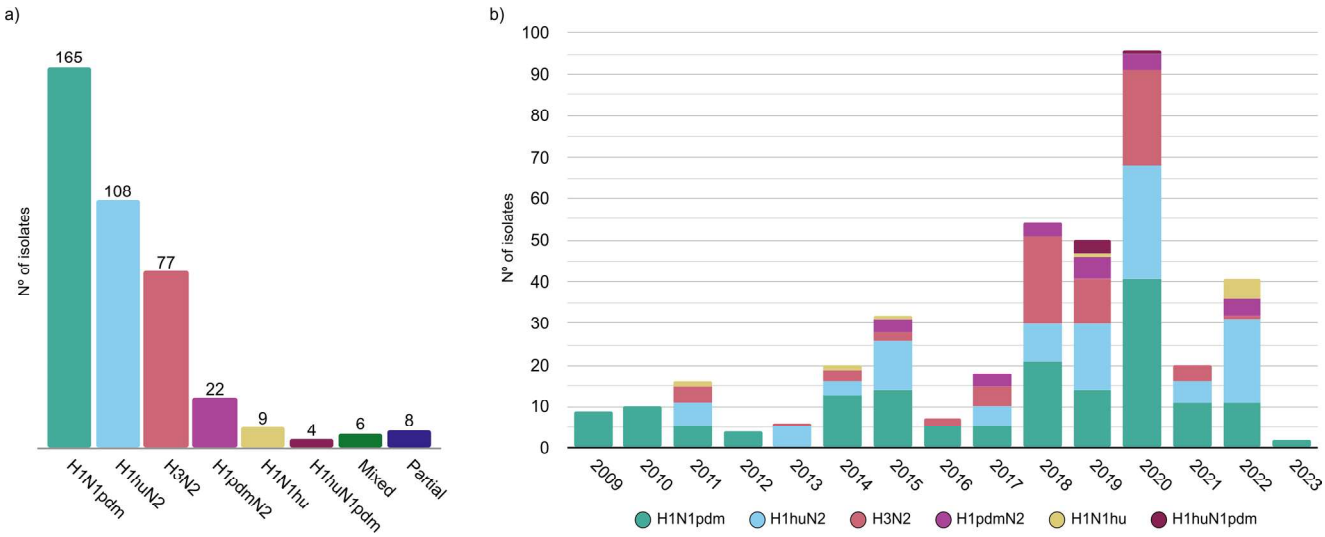

Fig. 1 Subtypes of influenza A viruses detected in Brazilian swine from late 2009 to early 2023. (**a**) Overview of all subtypes and lineages detected throughout the study period, including the samples that were partially subtyped and those in which two subtypes were found in the same isolate (mixed infection). (**b**) Subtypes and lineages detected annually. Results of partial subtyping and mixed detections are not shown for clarity and visual simplicity. Full data is presented in the manuscript

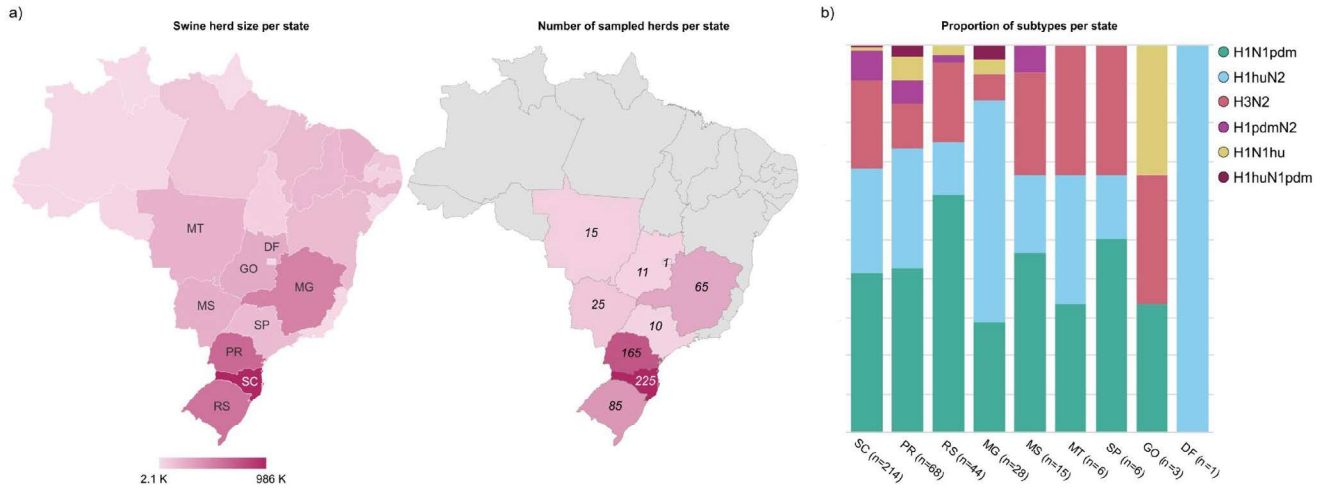

Fig. 2 Subtypes of swine IAV detected in Brazilian states included in this study. (**a**) Swine herd size per state in Brazil (year: 2023) and number of sampled herds during the study period. The color gradient is proportional to the number of pigs in each state. States with largest swine herds, such as Santa Catarina (SC), are shown in darker shades. The scale ranges from 2.1 to 986 thousand pig heads. (**b**) Proportion of IAV subtypes detected in each state shown in panel a. Partial subtyping results and mixed detections are excluded for visual simplicity but are presented in Table 1. State abbreviations: PR, Paraná; RS, Rio Grande do Sul; MG, Minas Gerais; MS, Mato Grosso do Sul; MT, Mato Grosso; SP, São Paulo; GO, Goiás; DF, Distrito Federal

Table 1 Subtypes and lineages of influenza A virus detected by state from 2009 to 2023

State of Brazil	Subtyped samples/Total of samples	Influenza A virus subtypes and lineages									
		H1N1			H1N2		H3N2[f]	Mixed	Partial		
		H1N1pdm[a]	H1N1hu[b]	H1huN1pdm[c]	H1huN2[d]	H1pdmN2[e]			H3	N1pdm	N2
Santa Catarina (SC)	222/353	88	2	1	58	16	49	3	2	1	2
Paraná (PR)	71/171	29	4	2	21	4	8	1	0	2	0
Rio Grande do Sul (RS)	46/88	27	1	0	6	1	9	2	0	0	0
Minas Gerais (MG)	29/66	8	1	1	16	0	2	0	0	1	0
Mato Grosso do Sul (MS)	15/27	7	0	0	3	1	4	0	0	0	0
Mato Grosso (MT)	6/16	3	0	0	1	0	2	0	0	0	0
Goiás (GO)	3/11	1	1	0	0	0	1	0	0	0	0
São Paulo (SP)	6/10	2	0	0	2	0	2	0	0	0	0
Distrito Federal (DF)	1/1	0	0	o	1	0	0	0	0	0	0
Total	**399/743**	**165**	**9**	**4**	**108**	**22**	**77**	**6**	**2**	**4**	**2**

[a]H1N1pdm: HA and NA derived from pandemic 2009 H1N1 virus.

[b]H1N1hu: HA and NA derived from pre-2009 human seasonal H1N1 influenza virus.

[c]H1huN1pdm: HA derived from pre-2009 human seasonal H1N1 influenza virus and NA derived from pandemic 2009 H1N1 virus.

[d]H1huN2: HA and NA derived from human seasonal H1N2 influenza virus.

[e]H1pdmN2: HA derived from pandemic H1N1 virus and N2 derived from human seasonal H1N2 or H3N2 influenza virus.

[f]H3N2: HA and NA derived from human seasonal H3N2 influenza virus.

studies that showed the predominance of H1N1pdm infection in pigs in Brazil following human-to-swine influenza transmission event in 2009 [12, 13]. In 2011, novel IAV subtypes (H1huN2, H3N2, and H1N1hu) derived from human-to-swine transmission events began to be detected in pigs [15, 28], and reassortant viruses were detected for the first time in 2015 (H1pdmN2) and in 2019 (H1huN1pdm). As of 2017, the number of samples received from the Midwest and Southeast regions increased because of the expansion of collections carried out in these regions. In the same period, an increase in the frequency of detection of H1huN2 and H3N2 influenza viruses was observed. Unfortunately, the number and distribution of samples per state were uneven (ranging from 1 to 80 annually), hindering efforts to define the prevalence of viral subtypes and lineages. Nevertheless, our study highlighted the high frequency of detection of H1N1pdm in all years except 2013, where the number of samples received for analysis was very small. The multiplex RT-qPCR employed here identifies the different subtypes and lineages of swIAVs that circulate in Brazil, however due to the wide genetic diversity detected in swIAVs in the last years, this technique requires frequent updating [22, 29].

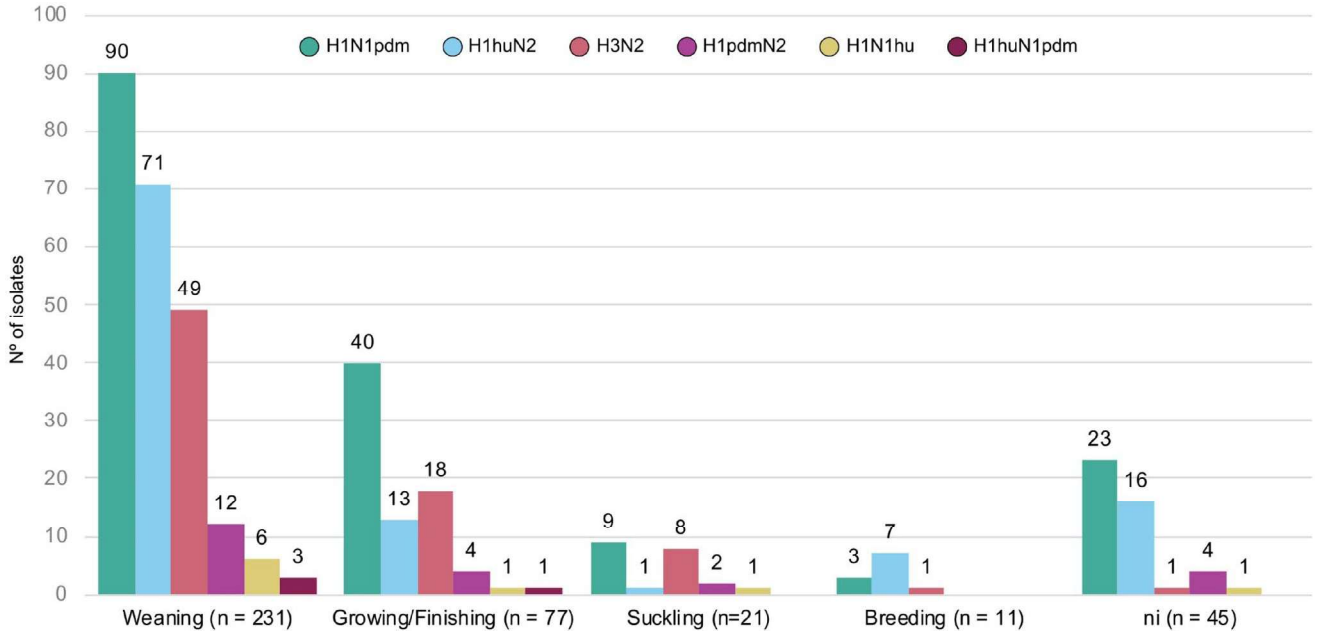

Fig. 3 Influenza A viruses and reassortants detected by production phase, from 2009 to 2023. Results of partial subtyping (8/399) and mixed detections (6/399) are not shown for clarity and visual simplicity. ni (not informed)

A noteworthy observation is that, shortly after the identification of H1N1pdm in pigs, other viral subtypes (H1huN2, H1N1hu, and H3N2) and reassortant viruses (H1pdmN2 and H1huN1pdm) began to be detected. Previous studies have observed a pattern for novel emerging swIAVs globally (including Brazil), with persistence in swine of HA and NA segments of human-seasonal virus origin and replacement of the internal gene segments through reassortment by H1N1pdm virus [30, 31]. The wide dissemination of IAV subtypes and lineages in different Brazilian states is remarkable and may reflect the transport of swine in Brazil for growth and finishing purposes. The long-distance swine movements have been associated with the spatial dissemination of human-origin H1 influenza viruses in North America pigs (from the Southern US to the Midwest), contributing to the introduction of multiple genetically diverse IAVs in those regions, providing the opportunity for genomic reassortment [32]. Despite the limited number of samples analyzed in Brazil, a significant genetic diversity of viruses circulating in pigs has been detected, with widespread dissemination across herds in the evaluated Brazilian states [15–18]. Human-origin influenza A viruses in swine have been detected globally, indicating ongoing transmission between pigs and humans [19, 20]. It is well established that seasonal human IAVs can be introduced into swine populations, where they may continue to circulate. These repeated introductions, especially involving multiple antigenically distinct HA and NA proteins, have significantly complicated the development of effective vaccines for controlling influenza in swine [30].

Detection of IAV per pig category was also evaluated, and most of the samples corresponded to weaning pigs, followed by growing-finishing, suckling piglets and breeding pigs. Growing pigs (21–63 days old) are considered highly susceptible to IAV infection, as maternally derived antibodies, although present, are waning at the time of transfer of piglets to the nursery, leading to limited immunity in the absence of vaccination [14, 33]. The control of influenza in weaned pigs is challenging, especially if pigs are infected at weaning and are raised in separate sites in distant locations, which contributes to regional dissemination of IAV [34]. In this study, the greatest viral diversity represented by the different subtypes and major lineages was identified in the group of weaned pigs (H1N1pdm, H1huN2, H3N2, H1pdmN2, H1N1hu, and H1huN1pdm). From 2019 (mainly 2020), an increase in the number of samples from finishing pigs was observed, with the detection of the three virus subtypes and their reassortants (H1N1pdm, H1huN2, H3N2, H1pdmN2, H1N1hu, and H1huN1pdm). Although the vaccination of sows in the country with autogenous vaccines began in 2017, pigs lacking pre-existing immunity continue to enter the finishing phase, as maternally derived antibodies do not persist until this stage [35].

Partial subtyping was obtained for 6.3% (25/399) of the virus strains analyzed. After sequencing, 2% of samples remained partially subtyped. Due to the mutation rate (antigenic drift) of the HA and NA genes, failures in the subtyping are expected to happen at some point and can be solved by primers updating from time to time [22, 29]. Even with a small number of virus strains analyzed here, detection

of reassorted viruses (6.5%) and mixed infections (1.5%), originated due to infection of the same cell by more than one IAV subtype or virus lineage, is notable and has been described by other authors [29, 35, 36]. In pig herds, the constant availability of non-immune piglets, together with practices of mingling piglets from various sources, favors the concomitant circulation of many different virus subtypes in herds, increasing the risk for co-infections and reassortments to occur [35, 37], and leading to the recurrence of IAV infection at the farm level [38]. This work is the result of years of effort by Embrapa and partners in monitoring swine influenza A in Brazilian herds. Although not designed to determine the prevalence of influenza infection, this study offers valuable insights into swIAV ecology in the country and underscores the need for the implementation of a national surveillance program. The great genetic diversity detected in swIAVs that are widely disseminated in Brazilian herds emphasizes the necessity of ongoing efforts to characterize swine viruses, update diagnostic tests and vaccines, and provide data critical for pandemic preparedness.

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Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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