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Evaluación y análisis antigénico de virus de campo como candidatos para la actualización del diagnóstico serológico de Influenza en cerdos en Colombia

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A mi mamá y a mi "pita", las mujeres de mi vida, por su incansable apoyo y amor incondicional. A mi hermana y mis sobrinos, por llenar mi vida de alegría. A mi abuelito, por sus consejos y oraciones. A Gloria Carrillo y Cristhian Galeano por sus palabras de aliento y por creer en mí.

"Somos dueños de nuestro destino. Somos capitanes de nuestra alma."

Winston Churchill

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Resumen

El Virus de Influenza A (VIA) en cerdos ocasiona infección respiratoria y se asocia al Complejo Respiratorio Porcino, generando impacto económico y sanitario. En Colombia, la limitada información sobre el VIA en cerdos ha llevado al uso de cepas de referencia internacional en pruebas de diagnóstico serológico. La representatividad de dichas cepas en el contexto colombiano es incierta, lo que afecta la precisión del diagnóstico nacional. Por lo tanto, el objetivo del presente estudio fue caracterizar molecular y antigénicamente VIA porcinos presentes en el país, elegir cepas representativas y verificar su utilidad como antígenos en pruebas serológicas. A través de aproximaciones basadas en secuencia y diversas metodologías moleculares, se caracterizaron 38 VIA provenientes de ocho regiones del país, detectados entre 2008 – 2021. Se eligieron siete candidatos virales, los cuales fueron evaluados mediante pruebas de Inhibición de Hemaglutinación (HI). Los resultados obtenidos muestran que en el país existe diversidad filogenética, lo que se evidencia por la identificación del subtipo H1N1 (clados 1A.3.3.2 y 1A.1) y con el reporte por primera vez de virus de los subtipos H1N2 y H3N2. Adicionalmente, se encontraron cinco clústeres antigénicos en el VIA H1 y se reconoció el H3 como una nueva variante genética y antigénica. Los siete candidatos elegidos permitieron una mejor detección de anticuerpos con respecto a las cepas de referencia utilizadas hasta el momento en el país, mostrando buena repetibilidad en la ejecución de la prueba de HI. A través de asociaciones estadísticas se logró reducir el número de candidatos propuestos a cuatro, los cuales pueden ser potencialmente usados como antígenos de referencia en las pruebas serológicas para VIA en cerdos en el país.

Palabras clave: Influenza porcina, caracterización antigénica, serología, clúster antigénico, bioinformática.

Abstract

Antigenic analysis of field viruses as candidates for the update of serological diagnosis of Influenza in swine in Colombia.

Influenza A Virus (IAV) in pigs causes respiratory infections and is associated to the Porcine Respiratory Disease Complex, accounting for economical loses and sanitary issues. In Colombia, knowledge about swine IAV is scarce. This has led to the implementation of international reference strains for serological assays. Representativity of these strains in the Colombian context is unknown affecting the national diagnostic capacity. Therefore, this study aimed the molecular and antigenic characterization of swine IAV in the county, the selection of representative strains, and the assessment of their value as antigens for serological assays. 38 IAV from eight regions of the country detected between 2008 – 2021 were characterized through sequence-based approaches. Seven candidates were selected for serological evaluation using the Hemagglutination Assay (HI). The results showed that there is a significant phylogenetic diversity in the country. This is supported by the recognition of the H1N1 subtype (clades: 1A.3.3.2 and 1A.1) and the identification for the first time of the H1N2 and H3N2 virus subtypes. Five antigenic clusters were recognized in the IAV H1 subtype as well as one in the H3 subtype representing a novel genetic and phylogenetic variant. The seven selected candidates allowed a higher antibody detection than the reference strains used until now in the country and they exhibited good repeatability among performs of the HI assay. Based on statistical associations the number of proposed candidates could be reduced to four. These candidates can potentially be included in serological assays in the country.

Keywords: Swine Influenza, Antigenic characterization, Antigenic cluster, Bioinformatics.

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Introducción

La influenza es una enfermedad viral de distribución mundial, que afecta a los cerdos impactando de manera negativa los sistemas de producción. Su presencia afecta el estatus sanitario de las granjas siendo además una patología que acarrea importantes pérdidas económicas y riesgos en materia de salud pública (Janke, 2013; Olsen et al., 2006a; Saade et al., 2020). El agente causal es un virus del género *Alphainfluenzavirus* o Virus de Influenza tipo A (VIA), el cual posee un genoma de ARN de cadena sencilla y polaridad negativa que se organiza en ocho segmentos independientes, lo que le confiere una alta capacidad de variación por procesos de mutación y reordenamiento genético (Drift y Shift, respectivamente) (ICTV, 2021). El virus se clasifica en Subtipos de acuerdo con la combinación de las glicoproteínas de superficie Hemaglutinina (HA) y Neuraminidasa (NA), de las que se reconocen hasta el momento 18 HA y 11 NA diferentes, afectando múltiples especies de aves y mamíferos, incluyendo al humano.

La infección en cerdos típicamente es ocasionada por los subtipos H1N1, H3N2 y H1N2, en los cuales se reconocen múltiples linajes y clados filogenéticos. La influenza en cerdos se presenta en forma de brotes agudos de enfermedad respiratoria caracterizada por alta morbilidad, mortalidad y recuperación variable dependiendo de la presencia de co-infecciones o infecciones bacterianas secundarias (OIE, 2009; Sugimura et al., 1981; van Dreumel & Ditchfield, 1968). El virus se replica en el epitelio del tracto respiratorio donde induce lesiones de bronconeumonía, consolidación pulmonar, infiltración de células inflamatorias y necrosis del epitelio bronquial y bronquiolar (Arenales et al., 2022; Janke, 2013; Watanabe et al., 2012).

La continua circulación del VIA es responsable de pérdidas económicas asociadas a costos de tratamiento de animales enfermos y afectación de parámetros productivos que resultan en sobrecostos en producción para la explotación. Se ha demostrado que en granjas positivas al VIA los animales presentan menor ganancia de peso y tasa de crecimiento; mayores tasas de mortalidad anuales, lo que implica una permanencia de los animales en

granja más larga, mayores gastos en alimentación, menos toneladas de carne producida por ciclo y por consiguiente menores ingresos (Calderón Díaz et al., 2020; Donovan, 2005; Haden et al., 2012). Adicionalmente, la presencia del VIA se relaciona con problemas de salud en granjas productivas dada su participación en el Complejo Respiratorio Porcino (CRP) (Brockmeier et al., 2002). En el CRP, el VIA interactúa con otros agentes induciendo cuadros de enfermedad severa y altas mortalidades. Se ha demostrado que agentes infecciosos como *Mycoplasma hyopneumonie*, *Streptococcus suis*, *Actinobacillus pleuropneumonie*, *Haemophilus parasuis* y *Pasteurella multocida* tienen relaciones sinérgicas con el virus al aumentar la severidad de las lesiones, el nivel de replicación viral y su tiempo de excreción, al igual que la expresión de genes proinflamatorios (Czyzewska-Dors et al., 2017; Dang et al., 2014; Deblanc et al., 2012; Pomorska-Mól et al., 2017). En contraste, la coinfección con otros agentes virales tiene efectos más variados, dadas las posibles relaciones de competencia, inhibición o sinergismo que pueden darse. Por ejemplo, se ha demostrado que la coinfección con virus como el del Síndrome Respiratorio y Reproductivo Porcino (PRRS) o el Circovirus Porcino tipo 2 (PCV-2) tiene un efecto sinérgico que además de exacerbar los cuadros de enfermedad respiratoria, disminuye la eficacia de la inmunidad inducida por vacunación contra estos agentes (Chrun et al., 2021; Sun et al., 2021).

Por otra parte, como resultado de la naturaleza zoonótica del virus y su potencial pandémico, la continua permanencia del VIA en los sistemas productivos constituye una amenaza para la salud pública mundial. Diferentes estudios muestran la capacidad del VIA de origen porcino de infectar a los humanos (Deng et al., 2020; Freidl et al., 2014; Parys et al., 2021), lo cual lo convierte en un agente con potencial pandémico y lo sitúa entre las fases 1-3 del riesgo de aparición de una pandemia por Influenza (WHO, 2009). Tal potencial quedó demostrado con la emergencia del clado pandémico del virus de influenza H1N1 en 2009 (H1N1pmd09; clado 1A.3.3.2). Una situación en materia de salud que remarcó nuevamente la importancia de los porcinos en la ecología del virus (Neumann et al., 2009). El H1N1pdm09 ocasionó consecuencias graves en la población humana y la economía global, al ser responsable de numerosas muertes, pérdidas económicas y sobrecostos en los sistemas de salud (Higgins et al., 2011; Rassy & Smith, 2013; WHO, 2013). Se estima que el virus acarreó pérdidas cercanas a los 22.800 millones de pesos a la industria porcina nacional en Colombia en 2009 (Asociación Colombiana de Porcicultores & Fondo Nacional de la porcicultura, 2009).

Actualmente el principal factor de riesgo en la infección por VIA en porcinos radica en el movimiento de animales entre granjas, ya que favorece la mezcla de grupos de diferente origen con estado sanitario muchas veces desconocido (J. T. Cheung et al., 2022; Olsen et al., 2006b). También se reconoce que las dinámicas poblacionales de los animales en subgrupos, dentro de los sistemas productivos, favorecen la persistencia de individuos susceptibles donde el virus se mantiene en replicación y evolución, siendo los grupos de mayor relevancia las hembras de reemplazo y los lechones neonatos (Chamba Pardo et al., 2018; Diaz, Marthaler, Culhane, et al., 2017; Diaz, Perez, et al., 2015; Ryt-Hansen et al., 2022). Dado esto, el control del VIA se centra mayormente en la aplicación de medidas de bioseguridad, la evaluación del estatus sanitario de los animales que ingresan y salen de las granjas, buenas prácticas de manejo y en algunas partes del mundo, protocolos de vacunación (Chamba Pardo et al., 2018; Ryt-Hansen et al., 2022; L. A. White et al., 2017). Sin embargo, además de la correcta aplicación de estas medidas, se requiere de técnicas de diagnóstico y monitoreo que permitan la identificación rápida y oportuna de individuos susceptibles, expuestos e infectados con el VIA.

La organización Mundial de Sanidad Animal (OMSA; anteriormente OIE) ha validado múltiples técnicas que permiten el diagnóstico del VIA en porcinos, de las cuales, las pruebas serológicas, basadas en interacciones antígeno-anticuerpo, han sido ampliamente usadas en el monitoreo y vigilancia del virus en los cerdos (WOAH, 2023). No obstante, considerando la amplia diversidad antigénica y alta variabilidad del VIA, se reconocen importantes dificultades en la implementación e interpretación de resultados de este tipo de pruebas. Por esta razón, la OMSA señala la necesidad de desarrollar, optimizar y evaluar las pruebas serológicas teniendo en cuenta las características antigénicas de los virus propios de cada región (OMSA, 2021; WOAH, 2023, 2024). Esta necesidad ha llevado a que países como Estados Unidos o los miembros de la Unión Europea implementen programas como el *National Surveillance Plan for Swine Influenza Virus in Pigs* o el *European surveillance network for influenza in pigs* (ESNIP) que han monitoreado a través de vigilancia pasiva y activa la circulación y evolución del virus, con la finalidad de generar información que contribuya, entre otras cosas, a la obtención de reactivos biológicos útiles en la actualización y optimización de pruebas diagnósticas, así como en el desarrollo de vacunas y estrategias de preparación frente a eventos potencialmente pandémicos (Simon et al., 2014; USDA, 2010).

En Colombia, esta directriz no se cumple a cabalidad dado que para el diagnóstico serológico históricamente se han utilizado antígenos de referencia internacional. Un hecho a considerar puesto que la capacidad diagnóstica del país se ve comprometida ya que la representatividad de los antígenos internacionales utilizados en el contexto nacional es incierta; más aún teniendo en cuenta que las características antigénicas de los virus circulantes en campo son desconocidas. La evidencia serológica señala que el VIA ha circulado en el país por al menos cincuenta años, evidenciándose reactividad serológica para los subtipos H1N1 y H3N2. Los primeros estudios indican seropositividad a virus de Influenza de los linajes H1N1 clásico y H3N2 en cerdos desde 1971 (Hanssen et al., 1977a), siendo este un panorama que se mantuvo por cerca de cuarenta años. La dinámica viral en el país evolucionó rápidamente con la introducción del virus H1N1pdm09 en 2009 (Flórez Ramos et al., 2018; Osorio-Zambrano et al., 2022; Ramirez-Nieto et al., 2012), bajo un patrón dependiente de su ubicación, por lo que hay evidencia de la aparición de subclados filogenéticos diferentes en áreas geográficas particulares (Osorio-Zambrano et al., 2022).

Considerando que el VIA ha mostrado evolucionar formando nuevos grupos antigénicos (D. J. Smith et al., 2004) y hay evidencia de su rápida evolución en el país tras la introducción del virus del tipo H1N1pdm09 (Osorio-Zambrano et al., 2022), se reconoce la necesidad de evaluar las características antigénicas de los virus circulantes en la región. Lo anterior, dado que es altamente probable que en Colombia los virus de Influenza hayan evolucionado en grupos con características antigénicas y genéticas particulares, como se ha observado en otras partes del mundo, en especial, teniendo en cuenta que en el país existe una presión de selección inmunológica diferente como consecuencia de la ausencia de vacunación frente a la que existe en otros países donde se emplean vacunas para inmunizar a los cerdos (T. K. Anderson et al., 2015; Chastagner et al., 2020; Lewis et al., 2016; Walia et al., 2019).

Por tanto, el principal objetivo de este trabajo es caracterizar molecular y antigénicamente VIA que han sido detectados en el país, con el fin de elegir y producir virus semilla a partir de los cuales se puedan obtener antígenos como potenciales referentes del panorama antigénico nacional para ser usados en el diagnóstico serológico de la influenza en cerdos en el contexto nacional.

Los resultados de este estudio aportarán al conocimiento del VIA no solo a nivel nacional sino internacional al ser la primera caracterización antigénica basada en secuenciación de los VIA circulando en la población porcina del país. Además, contribuirá a la generación de un banco de virus semilla que servirán como fuente de antígenos representativos del panorama viral que serán útiles no solo en pruebas serológicas, lo que robustecerá y mejorará las capacidades diagnósticas del país.

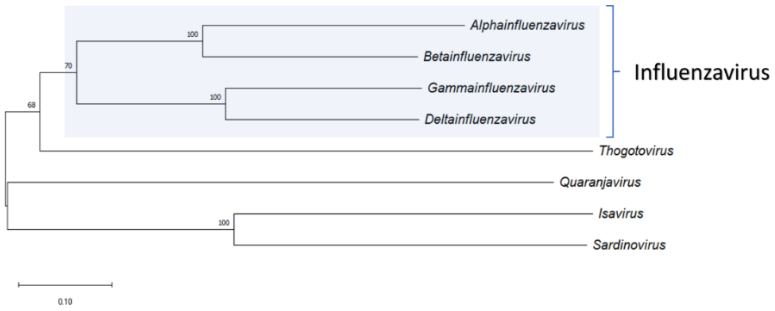
1. Marco teórico

1.1 El Virus de Influenza A

1.1.1 Taxonomía y clasificación de los virus de influenza

Los virus de Influenza pertenecen a la familia *Orthomyxoviridae,* la cual reúne diferentes virus que se caracterizan por tener un genoma de tipo ARN segmentado. Dentro de la familia, se reconocen los géneros *Alphainfluenzavirus* (Virus de Influenza tipo A)*, Betainfluenzavirus* (Virus de Influenza tipo B), *Gammainfluenzavirus* (Virus de Influenza tipo C) y *Deltainfluenzavirus* (Virus de Influenza tipo D) (Figura 1-1) (ICTV, 2021).

Figura 1-1 Virus de la Familia *Orthomyxoviridae*[1]



[1] Nueve secuencias del gen de la Polimerasa Básica 1 (PB1) representativas de cada género disponibles en el Genbank (https://www.ncbi.nlm.nih.gov/genbank/) fueron alineadas empleando el algoritmo MUSCLE en el Software MEGA 11.0 (https://www.megasoftware.net/) (Tamura et al., 2021). Su relación filogenética fue determinada por el método Neighbor-Joining en el Software MEGA 11.0 con un Bootstrap de 1000 repeticiones. Los números de acceso de las secuencias empleadas en la construcción del árbol fueron NC_026435, CY115117, NC_006308, NC_036615, NC_006503, NC_052682, MN241400 y NC_040731.

El *Alphainfluenzavirus* o Virus de Influenza A (VIA) es el más importante dentro de los Influenzavirus dado que ocasiona infección en un amplio rango de huéspedes que varía entre mamíferos y aves, al igual que cuadros de enfermedad respiratoria en humanos de manera global. Además, al ser un agente zoonótico con una rápida capacidad de adaptación y evolución, constituye una permanente amenaza a la salud pública siendo un virus con reconocido potencial pandémico (Long et al., 2019; Monto & Fukuda, 2020; M. Zhang et al., 2021). Este virus fue aislado por primera vez durante la década de 1930 tanto en animales como en humanos (Shope, 1931; W. Smith et al., 1933). Como consecuencia de su alta variación, es clasificado en subtipos según la combinación de las dos principales glicoproteínas de superficie: Hemaglutinina (HA) y Neuraminidasa (NA). Actualmente, se han descrito 16 subtipos de HA (H1-H16) y nueve de NA (N1-N9) que están presentes de forma natural en los reservorios aviares, al igual que dos subtipos de HA (H17-H18), dos de NA (N10-11) y un reciente H9N2-like presentes únicamente en quirópteros americanos y africanos, respectivamente (Kandeil et al., 2019; Tong et al., 2012, 2013). En cada subtipo, a su vez existen agrupaciones por linajes y clados filogenéticos que varían según la especie en la cual circule el virus (S. Liu et al., 2009; Zhuang et al., 2019). Los linajes se nombran con códigos alfanuméricos que pueden variar según el sistema propuesto. Ejemplo de esto son las clasificaciones hechas por Zhuang et al. (2019) para la agrupación de todos los subtipos de HA y NA en linajes universales, la de Anderson et al. (2016) que clasifica el subtipo H1 de cerdos en tres linajes o la de Smith & Donis (2014) que agrupa el linaje H5 del virus de influenza aviar de alta patogenicidad *A/goose/Guangdong/1/1996-like* en diferentes subclados.

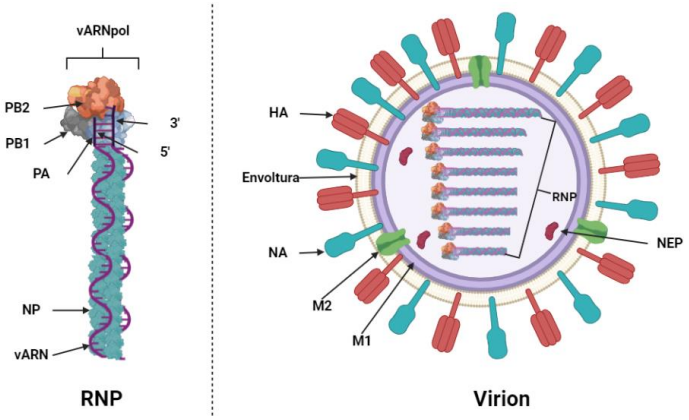
Respecto a los otros géneros, se ha identificado que estos tienen una menor variabilidad en comparación con el VIA, al igual que un menor rango de huéspedes. Los *Betainfluenzavirus* circulan mayormente de forma estacional en la población humana (Long et al., 2019) donde fueron identificados por primera vez en la primera mitad de la década de 1940 (Hannoun, 2013). El género se clasifica en dos mayores linajes: Victoria/2/87-*like* y B/Yamagata/16/88-*like*, en los que han sido descritos diferentes clados filogenéticos (R. Chen & Holmes, 2008). En cuanto a los *Gammainfluenzavirus* y *Deltainfluenzavirus*, se

sabe que difieren en su configuración genómica al presentar un segmento genómico menos que los otros tipos de Influenzavirus (ICTV, 2021). Estos dos géneros son considerados emergentes y tienen como huéspedes principales al humano y a los bovinos, respectivamente, aunque se han detectado en otros animales domésticos como el cerdo y otros rumiantes (Sreenivasan et al., 2021). Están subclasificados en seis linajes cada uno, siendo para el caso de los *Gammainfluenzavirus*: C/Kanagawa, C/Yamagata, C/Aichi, C/Sao Paulo, C/Taylor, y C/Mississippi; y para los *Deltainfluenzavirus:* D/OK, D/660, D/Yama2016, D/Yama2019, D/CA2019 y D/Bursa2013, (W. T. He et al., 2021; Yesilbag et al., 2022; W. Zhang et al., 2019).

1.1.2 Estructura

Las partículas del VIA son pleomórficas con formas redondeadas o filamentosas y tamaños que varían entre 80-120 nm o más de 20 µm, respectivamente. Los viriones presentan una envoltura lipídica que proviene de las células infectadas donde se ubican homotrímeros y homotetrámeros de las glicoproteínas HA y NA respectivamente, formando proyecciones similares a espículas (Figura 1-2). En la envoltura también está presente un canal iónico conformado por la proteína M2 y en la cara interna, la proteína de matriz (M1). En el interior, se ubican los segmentos de ARN del genoma viral encapsidados por su asociación a múltiples copias de una Nucleoproteína (NP), formando complejos ribonucleoproteícos (RNP), a los que a su vez se asocian tres proteínas (PB2, PB1 y PA) que conforman el complejo ARN polimerasa dependiente de ARN viral (vARNpol) (Figura 1-2) (Choppin et al., 1960; Noda & Kawaoka, 2010; Slater et al., 2022; Wrigley, 1979).

Figura 1-2: Representación del VIA y un complejo Ribonucleoproteíco[2]



1.1.3 Genoma y replicación

El genoma del VIA es de tipo ARN de cadena sencilla (ssARN) de polaridad negativa que se organiza en ocho segmentos independientes que tienen un tamaño entre 0.8 - 2.3 kb (Tabla 1-1). Cada segmento, presenta 13 y 12 nucleótidos conservados en sus extremos 5' y 3' que son complementarios, por lo que se pliegan formando una estructura secundaria de doble hélice, determinando la configuración tridimensional de los complejos RNP necesaria para la replicación y transcripción (Figura 1-2 y 1-3) (Noda & Kawaoka, 2010; Robertson, 1979). Cada segmento tiene un marco de lectura canónico, aunque en algunos se ha demostrado la presencia de marcos de lectura alternativos (Pinto et al., 2021; Vasin et al., 2014). El virus codifica al menos 10 proteínas canónicas, no obstante, se reconoce la existencia de péptidos accesorios relacionados con diferencias en la patogenicidad del virus e interacción virus-célula (Tabla 1-1) (Dou et al., 2018; Kamal et al., 2018; Pinto et al., 2021; Pleschka, 2013; Vasin et al., 2014).

[2] vARNpol: Polimerasa de ARN viral; PB2: Polimerasa Básica 2; PB1: Polimerasa Básica 1; PA: Polimerasa Ácida; HA: Hemaglutinina; NP: Nucleoproteína; NA: Neuraminidasa; M1: Proteína de Matriz; M2: Proteína M2 (canal iónico); NS1: Proteína no estructural 1; NEP: Proteína de exportación nuclear; vARN: Hebra de ARN viral; 3': Extremo 3'; 5': Extremo 5'. Elaborado en BioRender.com

Figura 1-3: Representación estructural de los segmentos genómicos del VIA [3]

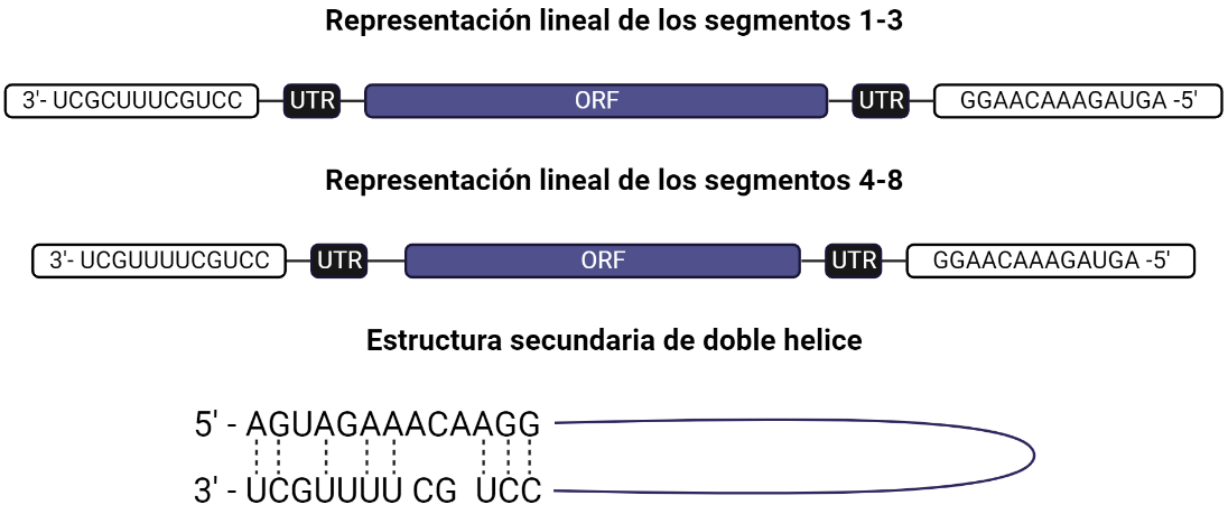


Tabla 1 - 1: Proteínas y péptidos accesorios codificados por cada segmento genómico[4]

Segmento genómico	Tamaño en bases	Proteína codificada	Función relacionada	Mecanismo involucrado
1	2.3 kb	PB2	Unión al CAP de mARN celular	Interacción con ARN pol
			Importación nuclear	Reclutamiento de importina-α
		PB2-S1*	Regulación de respuesta inmune	Interferencia en las vías RIG-I
2	2.3kb	PB1	ARN polimerasa	Sitio activo para la polimerasa
			Importación nuclear	Reclutamiento de importina-α
			Regulación de respuesta inmune	Interferencia en las vías RIG-I
		PB1-F2*	Pro-apoptosis	Inestabilidad mitocondrial
			Aumento de actividad de vARNpol	Retención nuclear de PB1
		PB1-N40*	Desconocida	Desconocido
3	2.2 kb	PA	CAP-endonucleasa	Asociación con PB2
			Importación nuclear	Reclutamiento de importina-α
		PA-X*	Interferencia en transcripción celular	Endonucleasa de mARN
		PA-N155*	Desconocida	Desconocido
		PA-N182*	Desconocida	Desconocido
4	1.7 kb	HA	Proteína de unión a receptor	Interacción con ácido siálico
			Fusión de membrana viral y endosomal	Péptido de fusión
5	1.5 kb	NP	Encapsidación del vARN	Unión a 12 nt de vARN
			Importación nuclear de RNP	Reclutamiento de importina-α
		eNP*	Patogenicidad viral	Desconocido
6	1.4 kb	NA	Liberación de nuevas partículas	Acción sialidasa en receptor
		NA43*	Desconocida	Desconocido
7	1 kb	M1	Exportación nuclear de RNP	Unión con NEP
			Ensamblaje del virión	Asociación con HA, NA y unión con RNP
		M2	Acidificación intraviral	Canal iónico
		M42*	Acidificación intraviral	Canal iónico

[3] En las cajas blancas se muestran las secuencias conservadas en los extremos 3' y 5, en las cajas negras las secuencias UTR (UnTranslated Region) y en las cajas moradas el marco de lectura ORF (Open Reading Frame) de cada segmento. Los segmentos se muestran con polaridad negativa (3' → 5'). Elaborado en BioRender.com con base en Breen et al. (2016); ICTV (2021); Robertson (1979) y Zhou et al. (2009).

[4] *Proteínas accesorias. Elaborado con base en: Dou et al., (2018); Kamal et al., (2018); Pinto et al., (2021); Pleschka, (2013); Vasin et al., (2014).

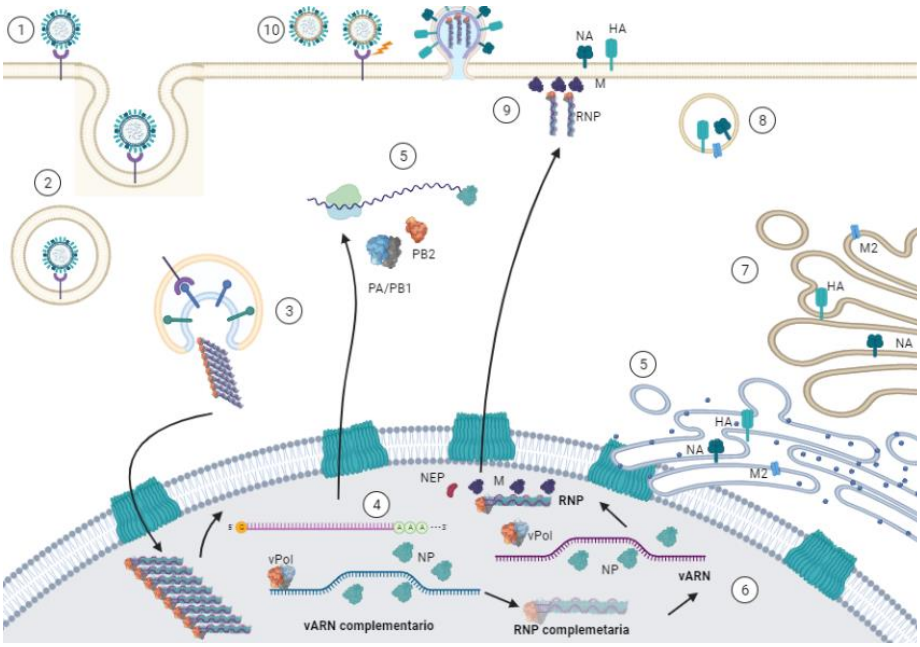
8	0.8 kb	NS1	Inhibición de interferón Exportación nuclear de mARN	Inhibidor de interferón Unión de transcritos a factores exportadores nucleares
		NEP	Exportación nuclear de RNP	Interacción con nucleoporinas y M1
		NS3*	Desconocida	Desconocido
		NSP*	Desconocida	Desconocido
		tNSP*	Regulación de respuesta inmune	Alteración en vías de interferón

El ciclo de infección se inicia con el reconocimiento de receptores celulares por parte de la HA, el cual se da a través de interacciones entre el Sitio de Unión a Receptor (RBD) presente en la región distal de la glicoproteína y moléculas de Ácido Siálico (AS) terminal presente en glicanos en la superficie celular (Figura 1-4). El AS puede estar presente en conformaciones α2-3Gal o α2-6Gal, que corresponden a las configuraciones predominantemente en aves y mamíferos, respectivamente (Anzaki et al., 2014; Fukuzawa et al., 2011). La unión de la HA con el receptor desencadena la internalización del virus en una vesícula endocítica que se forma a través de procesos complejos que pueden o no ser dependientes de clatrinas o caveolinas, en una reacción donde la concentración del Ca^{++} intracelular y la interacción adicional entre proteínas del virus y otras moléculas de superficie celular como la Nucleolina es esencial (Bao et al., 2021; C. M. Chan et al., 2016; Fujioka et al., 2013).

Para el correcto establecimiento de la infección celular, el material genético viral debe ser desensamblado de los viriones mediante la fusión de la membrana endosomal con la envoltura viral y el desacople de los complejos RNP de la proteína M1 (Figura 1-4), proceso que se lleva a cabo en una forma dependiente de pH. La fusión de membranas requiere la presencia de una HA previamente activada por el clivaje del precursor HA_0 en las subunidades HA_1 (región distal o globular) y HA_2 (región de tallo), puesto que es necesaria la exposición de un péptido de fusión en el extremo N-terminal de la HA_2. Este clivaje se da en el aminoácido 327 por la acción de proteasas celulares *Trypsin-like* y *TMPRSS2* (Bestle et al., 2021; Garten et al., 2015; Sriwilaijaroen & Suzuki, 2012). Tras la acidificación del endosoma, ocurre un cambio en la interacción molecular entre las proteínas del trímero de HA, lo que lleva a la orientación del péptido de fusión hacia la membrana endosomal fusionándola con la membrana viral, generando la formación de poros que permiten la salida de los complejos RNP (Figura 1-4). (Jakubcová et al., 2016). Para la correcta

liberación de los segmentos, los complejos RNPs y la proteína M1 deben disociarse, lo cual sucede igualmente como consecuencia de la acidificación dentro de la partícula viral (Bui et al., 1996; Chiang et al., 2017), en un proceso que es mediado por el paso de protones al espacio interior del virus a través de la proteína M2 activada por el bajo pH endosomal (Aledavood et al., 2022; Czabotar et al., 2004; Liang et al., 2016; Wharton et al., 1994)

Figura 1-4: Replicación del VIA[5]



[5] 1. El ciclo de infección se inicia con la unión de HA a receptores presentes en la superficie celular; 2. Tras la interacción de la HA con los receptores ocurre un proceso de endocitosis que permite al virus ingresar a la célula en una vesícula; 3. El genoma viral entonces sale de la vesícula endocítica a través de un proceso de fusión de membranas dependiente de pH que permite la salida de los complejos RNP al citoplasma celular; 4. Una vez los RNP se ubican en el citoplasma, son importados al núcleo donde se inicia la transcripción de mARN virales a partir del ARN genómico; 5. Los mARN virales salen del núcleo celular y se traducen en el citoplasma y retículo endoplasmático rugoso; 6. Para la replicación del genoma viral, las proteínas del complejo de replicación recién sintetizadas son importadas al núcleo donde soportan la síntesis de ARNs virales complementarios que sirven de plantilla para la síntesis de nuevas hebras de ARN genómico; 7. El ensamblaje se inicia con la maduración de glicoproteínas estructurales en el Aparato de Golgi; 8. Estas glicoproteínas son transportadas a la membrana celular donde forman regiones ricas en proteínas virales; 9. Los demás componentes virales se asocian a estas regiones en la membrana celular dando lugar a la formación de partículas virales; 10. Estas nuevas partículas son entonces liberadas por acción de la NA; vARN: ARN viral; RNP: Ribonucleoproteína; vPol: Polimerasa Viral; NP. Elaborado en BioRender.com

Una vez liberados en el citoplasma, los complejos RNP deben ser transportados al núcleo, proceso que es mediado por interacciones entre componentes de estos con los complejos de poro nuclear y proteínas de comunicación intracelular (Ling et al., 2022; Tome-Amat et al., 2019; H. Zheng et al., 2022). Ya en el núcleo, se inicia la transcripción, la cual ocurre en un proceso dependiente de cebador que precisa la toma de CAPs presentes en los mARN celulares, a través de una acción conjunta de las proteínas PB2 (captura de CAP) y PA (acción endonucleasa). Durante la toma del CAP, el mARN celular es cortado tomando 10-13 nucleótidos que aportan un extremo 3' (cebador) que es extendido por el sitio activo de PB1 y resulta en la síntesis de mARNs virales con CAP y poli A en los extremos 5' y 3', respectivamente (Dou et al., 2018; Pleschka, 2013; Te Velthuis & Fodor, 2016). Los mRNA virales son traducidos en el citoplasma (PB2, PB1, PA, M, NP, NS, NEP) y en el retículo endoplasmático rugoso (HA, NA, M2) proceso que es esquematizado en la Figura 1-4.

Para el proceso de replicación se requiere de proteínas PB2, PB1, PA y NP recién sintetizadas, las cuales deben ser importadas al núcleo (Dou et al., 2018). A diferencia de lo observado en la transcripción, la replicación ocurre en una forma independiente de cebador donde es crítica la estructura del complejo RNP y la de los extremos complementarios del ARN genómico. El proceso se inicia por la hibridación de dos nucleótidos trifosfato celulares con dos nucleótidos libres en los extremos 3' de los ARN virales (ARNv), que son unidos para formar un dinucleótido que posteriormente es extendido por PB1 y da lugar a ARNs complementarios (ARNc) con polaridad positiva, que son usados como plantilla para la síntesis del ARN genómico. Para poder ser utilizados como plantilla, los ARNc deben interactuar con nuevas proteínas NP para formar complejos RNP complementarios que son reconocidos por las proteínas PB2, PB1 y PA (Dou et al., 2018; Te Velthuis & Fodor, 2016).

A continuación, se da el ensamblaje de las nuevas partículas en la membrana celular donde se acumulan las proteínas HA, NA y M2 previamente maduradas en el aparato de Golgi (Dou et al., 2018; Pleschka, 2013). Los nuevos complejos RNP son transportados

fuera del núcleo y dirigidos al sitio de ensamblaje por la acción de las proteínas M1 y NEP (Brunotte et al., 2014; Neumann et al., 2000). Una vez localizados todos los componentes estructurales de los viriones en las regiones membranales ricas en HA y NA, se forman evaginaciones que dan origen a las partículas virales que son liberadas del anclaje a la membrana por una acción sialidasa de la NA (Figura 1-4) (Chlanda et al., 2015; Dou et al., 2018; Pleschka, 2013). Finalmente, la HA de las nuevas partículas es entonces clivada por enzimas membranales o extracelulares, quedando lista para iniciar nuevamente el proceso de infección viral.

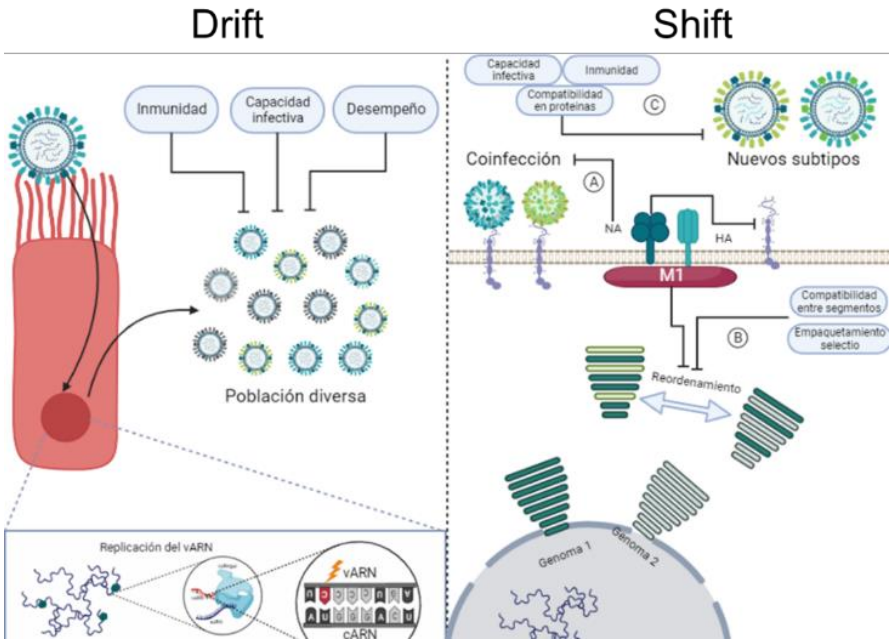
1.1.4 Mecanismos de evolución

Los principales mecanismos relacionados con la evolución en el VIA son la mutación (*Drift*) y el reordenamiento genético (*Shift*), y aunque la evidencia indica una alta frecuencia en la ocurrencia de estos eventos, las frecuencias observadas de ambos procesos son relativamente bajas. Esto se debe al impacto que los cambios generan en el desempeño viral (*Fitness*) con relación a su ambiente y el hospedero, por lo que cada variante del virus que se genere siempre estará sujeta a un proceso de selección. Por consiguiente, las frecuencias de mutación y reordenamiento observables corresponderán a aquellas variaciones que ofrecen alguna ventaja al virus, como la evasión de la respuesta inmune, o una replicación y transmisión más eficiente (Figura 1-5) (Domingo et al., 2021).

La variación por mutación o cambios menores ocurre durante cada ciclo replicativo como resultado de la aparición de errores en la secuencia de nucleótidos que son introducidos por la vARNpol en los diferentes segmentos, lo que resulta en la aparición de múltiples variantes virales (cuasi-especies o subpoblaciones) que aumentan la diversidad viral y ofrecen posibilidades de adaptación y evasión de la respuesta inmune (Figura 1-5) (Domingo et al., 1998; Ghorbani et al., 2020). La tasa de errores de la vARNpol ha sido determinada en múltiples ocasiones y bajo diferentes condiciones, siendo ésta considerada una enzima con una frecuencia de error elevada. Los primeros estudios como el de Parvin et al. (1986) señalan una tasa mutación de 1.5×10^{-5} nucleótidos por ciclo

infeccioso en el gen NS, lo que constituye una tasa mayor a la observada en otros virus con genoma de ARN. Análisis posteriores indican también que las mutaciones por base secuenciada o sustituciones de nucleótidos por hebra copiada se ubican en la magnitud mayor de 10^{-4} nucleótidos, siendo detectada la preferencia por cambios en las secuencias de A → G y U → C al igual que la existencia de un "*hot spot*" para sustituciones en el *motif* (A)AAG en secuencias copiadas experimentalmente por la vARNpol (P. P. H. Cheung et al., 2015; Pauly et al., 2017).

Figura 1-5 Mecanismos de evolución en el VIA y su regulación[6]



[6] Drift: Las variantes que emergen por errores durante la replicación del genoma son seleccionadas por diversos factores. Shift: Los principales factores reguladores del proceso de reordenamiento actúan en diferentes niveles durante el ensamblaje viral; a: durante el establecimiento de las coinfecciones; b: durante el ensamblaje; c: tras la liberación de los nuevos viriones. Elaborado en BioRender.com con base en Huang et al. (2008); Lowen, (2017); Richard et al. (2018); White et al. (2017); White & Lowen, (2018); Yen et al. (2011).

Los eventos de reordenamiento genético, por otra parte, pueden ocurrir naturalmente debido a la configuración segmentada del genoma viral y son una forma rápida de adaptación y de variación tanto genética como antigénica (Desselberger et al., 1978; Urbaniak et al., 2017). El reordenamiento se da cuando una célula es coinfectada por diferentes virus que, durante el ensamblaje, intercambian segmentos genómicos completos (Figura 1-5), lo que puede llevar a la emergencia de subtipos y variantes con nuevas características, como ocurrió con el virus del tipo H1N1pdm09 (Monto & Fukuda, 2020; York & Donis, 2012). Pese a que este proceso puede ocurrir con relativa frecuencia (De Silva et al., 2012; Marshall et al., 2013; Tao et al., 2014, 2015), el surgimiento de variantes reordenadas se observa en una frecuencia menor. Esto se debe a una serie de factores que determinan su establecimiento, incluyendo la necesidad de la coinfección, la cercanía evolutiva entre los virus, la sincronía en el ciclo replicativo, el tiempo entre una infección y la otra, la dosis infectiva de cada virus, la restricción anatómica de cada sitio de infección, la refractariedad en células infectadas por la destrucción de receptores en la membrana celular por acción de la NA, la compatibilidad de los segmentos genómicos, las señales de empaquetamiento selectivo de cada segmento, el balance entre las proteínas virales, así como la capacidad de transmisión, replicación y evasión de la respuesta inmune de cada nueva variante (Figura 1-5) (I.-C. Huang et al., 2008; Lowen, 2017; Richard et al., 2018; M. C. White et al., 2017; M. C. White & Lowen, 2018; Yen et al., 2011).

1.2 Determinantes antigénicos

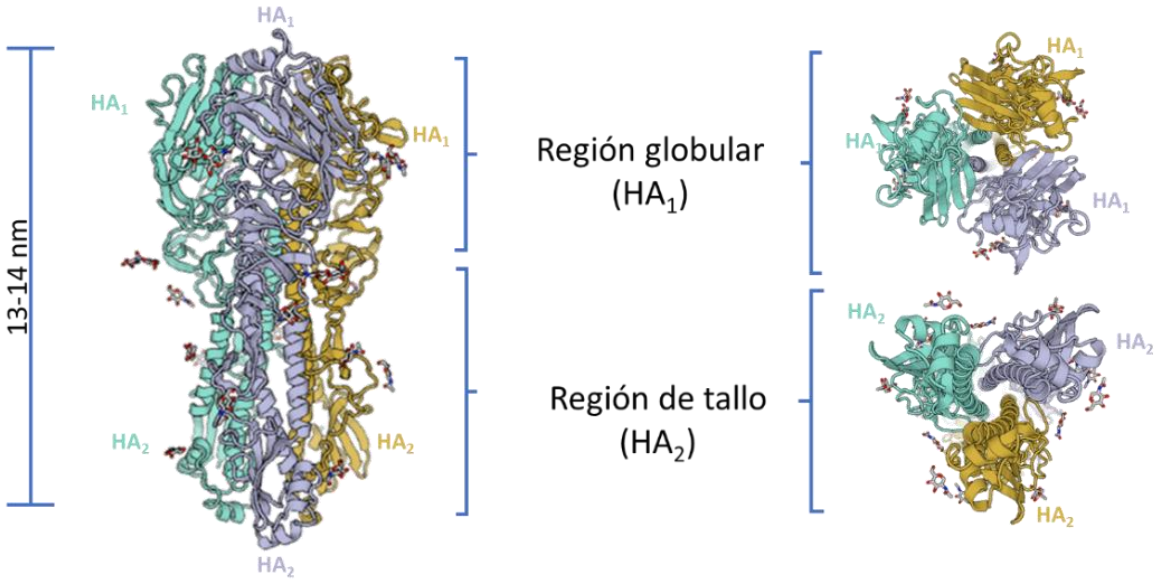
La utilización de la técnica de cartografía antigénica, la cual se basa en la capacidad de hemaglutinación de la HA y la capacidad inhibitoria (neutralizante) de anticuerpos dirigidos contra epítopes en esta, hace posible evaluar la diferencia antigénica entre las HAs de distintos VIA, al determinar la pérdida de reconocimiento por parte de un mismo grupo de anticuerpos frente a diferentes virus (Sitaras, 2020). Los resultados obtenidos mediante este análisis permiten medir y visualizar las distancias antigénicas en mapas de dos o tres dimensiones que agrupan los virus en clústeres o grupos antigénicos (Barnett et al., 2012). Pese a que la cartografía se considera el método de caracterización antigénica estándar,

existen importantes limitaciones en la técnica dado que solo se centra en la variación de regiones en la HA relacionadas con su actividad hemaglutinante. Por esta razón, la información respecto al perfil antigénico del VIA que se conoce actualmente únicamente representa ciertas características de la HA. Este hecho, ha llevado a que se reconozca la necesidad de investigar nuevas características antigénicas en la HA y en otras proteínas, lo que ha permitido identificar nuevos antígenos y determinantes antigénicos en el VIA.

1.2.1 Hemaglutinina

La HA constituye el antígeno inmunodominante en el VIA dada su relativa abundancia en la superficie viral (~5:1 en relación con NA) (Harris et al., 2006; Q. J. Huang et al., 2022), lo que la hace fácilmente reconocible por el sistema inmunológico. Estructuralmente, la HA consta de ~550 aminoácidos distribuidos en las subunidades HA_1 (~327 aminoácidos) y HA_2 (~222 aminoácidos). Ambas regiones son morfológicamente distinguibles en las proteínas una vez ensambladas en los viriones (Figura 1-6) (Q. J. Huang et al., 2022). Antigénicamente, la región de mayor relevancia es la HA_1, puesto que contiene el RBD y los principales epítopes; además, es la región donde se presentan con mayor frecuencia variaciones por *Drift* (Bizebard et al., 1995; Caton et al., 1982; Lees et al., 2011). La antigenicidad de la región del tallo, por otra parte, no fue considerada importante hasta que se descubrió su relativa estabilidad y capacidad de inducir una respuesta inmune protectora con reactividad cruzada entre variantes antigénicas e incluso entre subtipos diferentes del VIA (Ekiert et al., 2009; J. Huang et al., 2020; Yan et al., 2019). Este hecho la ha convertido en blanco de intensa investigación para el desarrollo de vacunas de amplio espectro (Bazhan et al., 2020; Corbett et al., 2019; Magadán et al., 2014), capaces de estimular la producción de anticuerpos con actividad inhibidora del péptido de fusión (Barbey-Martin et al., 2002). No obstante, la caracterización de la región del tallo resulta difícil y es poco aplicada de forma rutinaria, ya que requiere la utilización de pruebas más complejas como la seroneutralización (W. He et al., 2015).

Figura 1-6: Modelo estructural de un homotrímero de Hemaglutinina[7]



La propiedad antigénica de la HA se conoce desde las primeras investigaciones realizadas en el VIA, puesto que desde muy temprano se demostró que su variación afecta tanto el patrón antigénico como el *fitness* viral (Kilbourne et al., 1979, 1988; Meier-Ewert & Dimmock, 1970; Nath et al., 1975). Esto señala que en la glicoproteína existe una fuerte presión de selección inmunológica y funcional, que a lo largo de la historia evolutiva del virus ha resultado en la emergencia de variados grupos antigénicos y filogenéticos. La investigación en la emergencia de los nuevos clústeres antigénicos ha demostrado que la aparición de tales variantes ocurre a lo largo del tiempo en un patrón puntuado de “saltos”, donde cada nuevo clúster debe alcanzar un umbral de sustituciones suficiente para inducir cambios significativos en el perfil antigénico (D. J. Smith et al., 2004). La determinación del umbral que genera la emergencia de variantes antigénicas es algo difícil de medir, puesto que hay evidencia que indica que nuevos clústeres pueden resultar incluso del cambio de

[7] Adaptado del modelo de microscopía electrónica de la HA del virus A/Michigan/45/2015(H1) realizada por Boyoglu-Barnum et al., (2021) disponible en Swiss Model Template Library (https://swissmodel.expasy.org/templates/) identificado con el código 7kna.1 (Waterhouse et al., 2018).

un solo aminoácido dentro de la secuencia de la HA (D. J. Smith et al., 2004; C. Xu et al., 2022).

La incertidumbre respecto a la variabilidad antigénica de la HA ha llevado a que múltiples investigaciones propongan posiciones dentro de la secuencia de la proteína que presentan una mayor ponderación en el perfil antigénico. Consecuentemente, se ha logrado identificar los aminoácidos constituyentes de los mayores epítopes en la HA, los cuales han sido mapeados en la región globular donde se forman estructuras complejas al no ser epítopes lineales. Actualmente, los subtipos mejor caracterizados son los H1 y H3, en los que se reconocen cinco epítopes canónicos (epítopes Sa, Sb, Ca1, Ca2 y Cb en H1, y A, B, C, D y E en H3) que han servido de referentes en el estudio de otros subtipos (Brownlee & Fodor, 2001; Caton et al., 1982; Wiley et al., 1981; I. A. Wilson & Cox, 2003) y han permitido determinar que las regiones antigénicas varían entre subtipos de la HA. Tal es el caso del H2 en el que existen al menos seis epítopes (I-A, I-B, I-C, I-D, II-A y II-B), de los que solo tres tienen una posición compatible con las posiciones en H3 (Tsuchiya et al., 2001), al igual que en el subtipo H5, donde se han descrito tres regiones, de las cuales solo dos tienen posiciones equivalentes a los epítopes en H1 y H3 (Kaverin et al., 2002).

Si bien el conocimiento de los epítopes ha permitido entender y modelar la variación antigénica en la HA, la evolución observada naturalmente indica la existencia de "*hot spots*" en los que la mutación de ciertos aminoácidos aumenta considerablemente la distancia antigénica. Esto ha llevado a que las nuevas aproximaciones se centren en el desarrollo de modelos y algoritmos predictivos que permitan medir el impacto de las sustituciones de cada uno de los aminoácidos dentro de los epítopes (Quan et al., 2019; Xia et al., 2021). Estudios como el de Stray & Pittman (2012), señalan que la importancia de la variación aminoacídica en la conformación epitópica se debe a la alteración de las propiedades biofísicas en la HA, puesto que modifican la forma, la polaridad o la accesibilidad de los epítopes e interfiere con el acceso y las fuerzas energéticas necesarias durante la interacción con anticuerpos.

Otro factor importante para considerar en la antigenicidad de la HA es que la existencia de modificaciones postraduccionales puede igualmente afectar la estructura tridimensional de la glicoproteína, siendo mayormente reconocida la importancia de la N-Glicosilación, aunque también existen otras modificaciones como la palmitoilación (Hu et al., 2020). La adición de glicanos en la HA, es un proceso que se da durante su maduración en el aparato de Golgi y su ganancia o pérdida ha demostrado influenciar la estructura y las propiedades electroestáticas de la HA haciéndola antigénicamente diferente (Chang & Zaia, 2019; Hause et al., 2012; Schulze, 1997). La N-glicosilación, ha demostrado igualmente ser relevante en la variación antigénica en epítopes presentes en la región del tallo (Magadán et al., 2014), por lo que durante la evaluación antigénica de esta proteína se hace necesario considerar siempre la presencia de *motifs* de N-glicosilación.

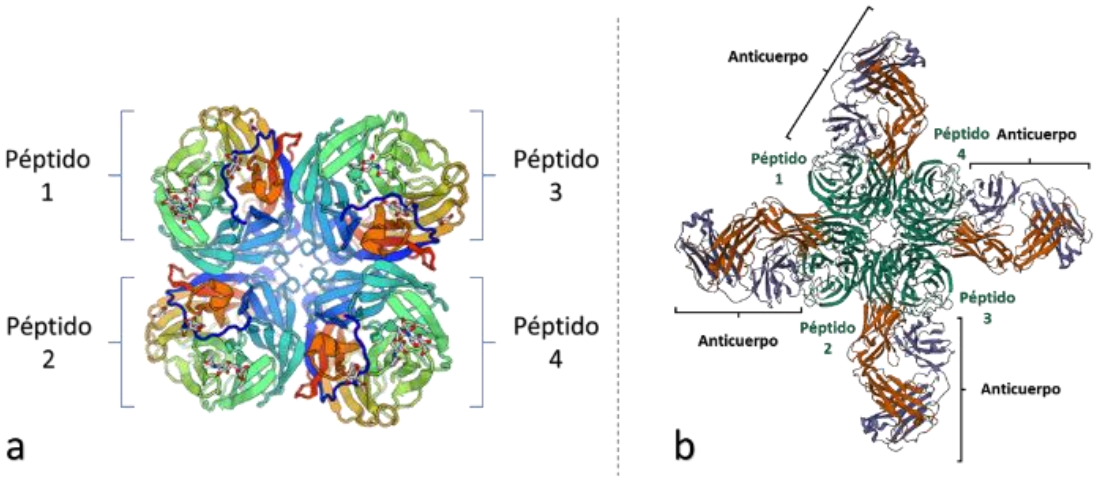
Con la aparición y el avance de las ciencias bioinformáticas han emergido nuevas formas de caracterización antigénica de la HA, varias de ellas basadas en la predicción de epítopes que son reconocidos por células linfocíticas T ($CD4^+$ y $CD8^+$) (Gutiérrez et al., 2017; Renu et al., 2020). Muchos de los epítopes son establecidos por algoritmos que determinan la existencia de *motifs* de unión entre la HA y los alelos del Complejo Mayor de Histocompatibilidad (CMH) (E. J. Grant et al., 2014). Actualmente, de acuerdo con el *Immune Epitope Database and Analysis Resource* (www.iedb.org), existen al menos 799 epítopes identificados en la HA que son reconocidos por células T (accedido el 22 de enero de 2024).

Otras aproximaciones bioinformáticas más conservadoras también han sido desarrolladas. En estas metodologías el análisis antigénico del VIA se ha centrado en la identificación de variaciones en los epítopes canónicos. Dicha variación es medida y visualizada a través de procesos matemáticos que permiten estimar con una buena exactitud distancias antigénicas, clústeres antigénicos y efectividades vacunales (C. S. Anderson et al., 2018; V. Gupta et al., 2006; M. S. Lee et al., 2007; Pan et al., 2011).

1.2.2 Neuraminidasa

El segundo mayor determinante antigénico en el VIA es la NA, puesto que al igual que la HA, es fácilmente reconocida por el sistema inmunológico. A diferencia de la HA, la NA presenta *Drift* en una frecuencia menor y de forma independiente (Sandbulte et al., 2011), lo cual puede deberse a una abundancia más baja en la superficie viral (Q. J. Huang et al., 2022) y por consiguiente a una menor presión de selección. Estructuralmente la glicoproteína se ensambla como homotetrámeros compuestos de péptidos que, según el subtipo, pueden ser de entre 452-471 aminoácidos. Cada péptido se organiza topográficamente en cuatro dominios, denominados: de cabeza, de tallo, transmembranal y citoplasmático. El dominio más estudiado y por consiguiente mejor caracterizado es el de cabeza (análogo a la región globular de HA) (Figura 1-7a), en el cual se encuentran los principales sitios de reconocimiento antigénico (Creytens et al., 2021). En esta región se ubica el sitio activo responsable de la acción catalítica de la NA y un sitio de reconocimiento de receptores de AS, que se encarga de la “captura” del AS previo a su catálisis. La región de cabeza, a diferencia de lo observado en la región globular de la HA, parece ser relativamente conservada, lo que refleja la importancia de su función biológica para el *fitness* viral (Sylte & Suarez, 2009). Como es de esperar, el sitio activo y el de unión a receptores son los de mayor conservación, aunque existe evidencia de que se necesitan cambios en estos durante la adaptación del VIA a nuevos huéspedes (Du et al., 2018, 2021). La región de tallo, por otra parte, presenta una considerable variabilidad por la existencia de deleciones y la adquisición de modificaciones postraduccionales (Creytens et al., 2021; Park et al., 2017; Sylte & Suarez, 2009; H. Zhou et al., 2009).

Figura 1-7: Modelo estructural del dominio de cabeza de un homotetrámero de NA[8]



La antigenicidad de la NA es menos entendida que la de la HA y sus características han sido históricamente ignoradas durante el desarrollo de biológicos contra el VIA. No obstante, desde la década de 1980 diferentes grupos han demostrado la existencia de sitios antigénicos que pueden tener o no una capacidad neutralizante de la NA (Figura 1-7b). La descripción de estos sitios varía según la metodología y los análisis realizados. Colman et al. (1983), por ejemplo, señala que en la NA del subtipo N2 existen siete sitios antigénicos (I-VII), en tanto que Webster et al. (1984) y Jackson & Webster (1982) proponen la existencia de entre tres y cuatro sitios sobrelapantes. Otros estudios en este mismo subtipo proponen que, según análisis evolutivos, existen clústeres de aminoácidos hipervariables que podrían contribuir en mayor medida a la evasión del reconocimiento antigénico (Laver et al., 1982). Respecto a los otros subtipos, existe una pobre caracterización y los reportes mayormente se limitan a la detección de regiones

[8] a: Adaptado del modelo de difracción de rayos X de la región de cabeza de la NA del virus A/BrevigMission/1/1918(H1N1) realizado por Campbell et al. (2021) disponible en Swiss Model Template Library (https://swissmodel.expasy.org/templates/) identificado con el código 6d96.1 (Waterhouse et al., 2018). b: Adaptado del modelo de difracción de rayos X de la región de cabeza de la NA del virus A/Minnesota/11/2010(H3N2) en asociación a anticuerpos neutralizantes de la NA realizado por Yasuhara et al. (2022) disponible en el Protein Data Bank (https://www.rcsb.org) bajo el código de acceso 6N6B.

reconocidas por anticuerpos específicos sin señalar la composición completa de los epítopes (Wan et al., 2013, 2015; J. R. Wilson et al., 2016).

La inmunidad frente a la NA no neutraliza al VIA, pero ha demostrado tener un efecto protector frente a la infección, ya que el bloqueo de la glicoproteína ocasiona una liberación deficiente de las nuevas partículas virales (Creytens et al., 2021; Xiong et al., 2020; Yasuhara et al., 2022). Este principio aunado a la relativa estabilidad de la región de cabeza en la NA ha motivado la investigación en búsqueda de epítopes altamente conservados capaces de inhibir la acción de la NA. Esto ha resultado en el reconocimiento de un epítope universal presente en todos los subtipos de NA que al ser bloqueado por anticuerpos induce una menor actividad enzimática y afinidad por el AS (Doyle, Hashem, et al., 2013; Doyle, Jaentschke, et al., 2013; Stadlbauer et al., 2019). Este epítope se ubica entre las posiciones 222-230 y su conservación es el resultado de su importancia en la replicación viral, puesto que se ha demostrado que mutaciones en estas posiciones ocasionan afecciones a la integridad estructural de la NA y a su actividad biológica (Doyle, Jaentschke, et al., 2013).

Análisis de la evolución antigénica en la NA sugieren que en la glicoproteína existen “epítopes energéticos” dentro de las regiones antigénicas, en los que una sola variación impacta significativamente en el reconocimiento inmunológico (J. T. Lee & Air, 2006). Tal impacto, usualmente se debe a cambios en las cargas en los aminoácidos (Y. Wang et al., 2021). Hoy se sabe que algunos de los factores relevantes en el perfil antigénico de la NA son la ganancia o pérdida de N-glicosilación (Ge et al., 2022; H. Powell & Pekosz, 2020; Y. Wang et al., 2021), al igual que la sustitución de los aminoácidos específicos 328, 342, 356, 367, 369 y 390 (F. Wang et al., 2021; Y. Wang et al., 2021; Yasuhara et al., 2019). De igual manera, se conoce que la evolución antigénica de la NA ocurre mayormente en una sola dirección, caracterizada por un patrón en el que las nuevas variantes pueden tener reacción cruzada con anticuerpos desarrollados contra variantes antiguas, mientras que las variantes antiguas no presentan reactividad cruzada con anticuerpos desarrollados frente a variantes recientes (Gao et al., 2019a).

La caracterización de la NA se basa en pruebas de Inhibición de la Neuraminidasa (NI) que buscan determinar el nivel de actividad catalítica de la glicoproteína cuando se enfrenta a diferentes grupos de anticuerpos. Las pruebas miden la actividad enzimática a través de reacciones químicas que detectan la exposición de galactosas terminales o la cantidad de AS libre que se genera por su actividad (Pedersen, 2008). Para los análisis antigénicos actualmente se emplean técnicas de tipo ELLA (*Enzyme-Linked Lectin Assay*) (Couzens et al., 2014; Gao et al., 2016; Pedersen, 2008; Sandbulte et al., 2009). Al igual que en la HA, la aparición de nuevas tecnologías ha guiado el estudio de la NA hacia la predicción de sitios antigénicos con base en secuencias que identifican potenciales epítopes reconocidos por células inmunes (Almalki et al., 2022; S. K. Gupta et al., 2011; Renu et al., 2020). De momento, de acuerdo con el *Immune Epitope Database and Analysis Resource* (www.iedb.org), existen al menos 180 epítopes potenciales que son reconocidos por células T (accedido el 22 de enero de 2024).

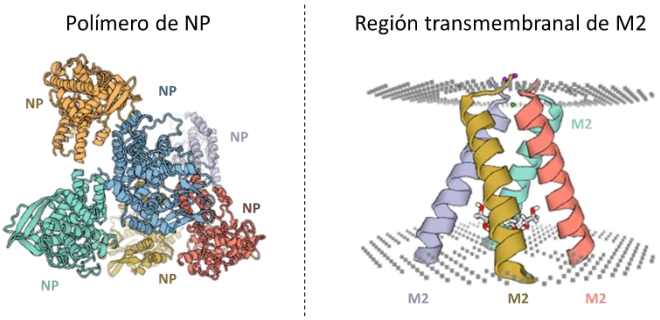
1.2.3 Otras proteínas como determinantes antigénicos menores

Otros determinantes antigénicos importantes en el VIA son las proteínas internas M1, M2 y NP, que han demostrado ser más conservadas que las de superficie y contribuyen a la generación de inmunidad frente a un amplio rango de subtipos (Holzer et al., 2019). La propiedad conservada de estos antígenos ha sido la base en el desarrollo de pruebas serológicas que detectan la exposición al virus independientemente del subtipo, resultando en la existencia de múltiples kits comerciales de ELISA comúnmente usados en el monitoreo y la vigilancia del VIA en diferentes especies.

La NP es una proteína abundante en células infectadas y se organiza mayormente en forma de trímeros (Figura 1-8) que se polimerizan junto con el ARNv para la formación de los complejos RNP. Esta proteína es altamente conservada entre diferentes subtipos y ha sido empleada en la clasificación taxonómica de los diferentes géneros de los Influenzavirus. En la NP se han identificado epítopes lineales y conformacionales que

inducen respuestas de tipo humoral (Gui et al., 2014) y experimentalmente se ha comprobado la existencia de al menos seis que inducen respuestas celulares (E. Grant et al., 2013). Estudios evolutivos demuestran la existencia de un sitio con una alta presión de selección en los aminoácidos 147 – 155 que interesantemente se relacionan con un sitio de activación de respuestas celulares citotóxicas, lo que sugiere que a diferencia de lo observado en HA y NA, la presión sobre esta proteína es principalmente mediada por la inmunidad celular (McGee & Huang, 2022). Este hecho, sin embargo, no implica que la respuesta humoral a la NP sea despreciable, puesto que se conoce que la respuesta de anticuerpos frente a esta puede conferir algún nivel de protección a la infección por diferentes subtipos, en especial cuando se logra una respuesta celular simultánea que además involucra a la proteína M2 (Bazhan et al., 2020; Mytle et al., 2021; Rostaminia et al., 2022; W. Wang et al., 2019; M. Zheng et al., 2014). Además, la utilidad de la respuesta humoral a NP ha servido ampliamente en la detección de infecciones por VIA de diferentes orígenes (Tu et al., 2019) e incluso, se ha postulado que a través del estudio de variaciones en los aminoácidos es posible identificar marcas o firmas en NP específicas de subtipos (Miyoshi-Akiyama et al., 2012). La proteína M2 por su parte no es tan abundante en los viriones y se organiza en homotetrámeros que atraviesan la membrana viral (Figura 1-8). Su estudio se ha centrado mayormente en relación con antivirales como la amantadina (Moorthy et al., 2022). Sus características antigénicas se limitan principalmente al ectodominio expuesto en el cual se ha identificado que los aminoácidos presentes en las posiciones 10-24 impactan considerablemente en su polaridad y su perfil antigénico (Sumarningsih et al., 2019). En ambas proteínas se han identificado 537 y 38 regiones respectivamente, como potenciales epítopes que son reconocidos por células T (disponibles en http://www.iedb.org, accedido el 22 de enero de 2024).

Figura 1-8: Modelo de un polímero de NP y la región transmembranal de M2[9]



1.3 Virus de Influenza A en cerdos

1.3.1 El porcino como huésped del VIA

Los porcinos son susceptibles a VIA de origen aviar y humano, por lo que favorecen la presentación de eventos de reordenamiento y adaptación viral (Gong et al., 2021; Ma et al., 2009; Pinsent et al., 2016). Esta susceptibilidad ocurre por la gran diversidad de receptores del tipo AS que expresan, pudiendo encontrarse en la forma de Ácido N-acetilneuramínico (Neu5Ac) y Ácido N-glicolilneuramínico (Neu5Gc), en unión a galactosas en configuración α2,3Gal, reconocida por virus de tipo aviar y α2,6Gal, reconocida por virus humanos (Nelli et al., 2010; Suzuki et al., 1997; Trebbien et al., 2011). Esta variabilidad se debe a la expresión de enzimas como *CMAH* y *ST3GAL4* (Spruit et al., 2021; Zhao et al., 2021). Aun así, la probabilidad del establecimiento de infecciones en cerdos varía por

[9] Polímero de NP: Adaptado del modelo de microscopía electrónica de un polímero de cuatro proteínas NP de un virus de influenza A realizado por Moeller et al. (2012) disponible en Swiss Model Template Library (https://swissmodel.expasy.org/templates/) identificado con el código 2ymn.1 (Waterhouse et al., 2018). Región transmembranal de M2: Adaptado del modelo de difracción de rayos X de un tetrámero de M2 del virus A/Udorn/307/1972 realizado por Thomaston et al. (2015) disponible en el Protein Data Bank (https://www.rcsb.org) bajo el código de acceso 4qkl.1.

cuenta de la distribución y abundancia de cada configuración a lo largo del tracto respiratorio, al igual que su capacidad de interacción con la HA. El nivel de expresión de Neu5Gc varía entre el 9-53% (Spruit et al., 2021; Suzuki et al., 1997) y su interacción con el virus es limitada, apareciendo preferencialmente en ciertos subtipos portadores de mutaciones en HA como Y161A, en tanto que la forma Neu5Ac es ampliamente reconocida por la mayoría de los VIA (Bateman et al., 2010; Broszeit et al., 2019; Spruit et al., 2021). La distribución de α2,3Gal y α2,6Gal en el tracto respiratorio de cerdos se asemeja a la humana, con predominio del tipo α2,6Gal en el tracto superior y α2,3Gal en el inferior, estando presente también en la submucosa y glándulas del tracto respiratorio superior (Bateman et al., 2010; Spruit et al., 2021; Van Poucke et al., 2010; Zhao et al., 2021).

De manera adicional, la coexpresión de ciertas proteínas permite la eficiente replicación e infectividad del VIA, ya que durante la síntesis de la HA es posible su maduración por la acción enzimática de serin-proteasas transmembranales porcinas (*swTMPRSS2*) y la proteasa porcina *trypsin-like* de vías aéreas (*swAT*) (Peitsch et al., 2014). De igual manera, la expresión de *swANP32A* y *swANP32B* soportan la replicación de virus tanto de origen mamífero como aviar, siendo la única especie mamífera doméstica con esta característica que resulta de una variación en *swANP32A* (H. Zhang et al., 2020).

1.3.2 Historia de la influenza porcina

La historia de la influenza porcina se resume en cuatro eventos clave que llevaron al establecimiento del virus en la especie. El primer evento se remonta a Norteamérica durante la década de 1930, cuando se logró el primer aislamiento de un VIA (subtipo H1N1) a partir de tráquea y pulmones de cerdos que presentaban sintomatología respiratoria (Shope, 1931). Los cerdos habían sido diagnosticados con una enfermedad emergente que se reportó por primera vez en 1919 que, por su similitud con la influenza pandémica de la época, fue nombrada influenza porcina o "*hog flu*" (M. I. Nelson & Worobey, 2018; Schultz-Cherry et al., 2013). Mundialmente, se acepta que la pandemia de 1918 es el

origen de estos primeros virus detectados en cerdos; sin embargo, existe evidencia que podría sugerir la circulación del VIA en esta especie desde mucho antes y en otras partes del mundo (Morens & Taubenberger, 2014).

El segundo evento corresponde a los múltiples episodios de movimientos inter-especie de diferentes virus de la influenza humana que se dieron durante 1970-1998 y que resultaron en el establecimiento del subtipo H3N2 de forma independiente en al menos tres continentes (Asia, Europa y América). Los primeros virus del subtipo H3N2 fueron reportados en Asia en 1970, en Europa en 1984 y en América en 1998 (Brown, 2000; Kundin, 1970; Yu et al., 2008; Zell et al., 2013; N. N. Zhou et al., 2000). De igual forma, múltiples introducciones de virus H1N1 de origen estacional humano han llevado al establecimiento de virus *Human-like* filogenéticamente diferentes a través de eventos de zoonosis reversa reportados en múltiples partes del mundo (Brown, 2011; Chastagner et al., 2020; Lorusso et al., 2011).

El siguiente evento tuvo lugar en Bélgica en 1979 y constituye la primera evidencia de salto inter-especie desde aves a cerdos de forma natural. Este salto inter-especie dio lugar al establecimiento de un nuevo virus del subtipo H1N1 *Avian-like,* genética y antigénicamente diferente al H1N1 de 1931 norteamericano. La llegada de este nuevo virus fue seguida por su diseminación y dominancia en la región europea alcanzando a Asía solo 20 años después (M. I. Nelson et al., 2014; Pensaert et al., 1981; Zell et al., 2013; H. Zhu et al., 2013).

Finalmente, el evento más reciente se dio en 2009 cuando en Centroamérica se detectó la emergencia de un virus H1N1 reordenado, que fue responsable de la primera pandemia del presente siglo (H1N1pmd09). Este virus emergió como consecuencia del *Shift* entre diferentes VIA previamente detectados en cerdos y rápidamente se diseminó alcanzado poblaciones porcinas en todo el mundo como consecuencia de repetidas introducciones desde fuentes humanas (M. I. Nelson, Gramer, et al., 2012; Neumann et al., 2009). El nuevo virus tenía una configuración genómica que incluía genes de origen humano (PB1

– H3N2), aviar (PB2, PA), porcino de Norteamérica (NP, HA, NS – H1N1) y porcino de Europa (M, NA – H1N1) (Neumann et al., 2009; G. J. D. Smith et al., 2009).

1.3.3 Subtipos y linajes

A pesar de la susceptibilidad de los porcinos a diferentes subtipos del VIA, solo los subtipos H1N1, H1N2 y H3N2 se han logrado establecer de forma endémica en esta especie. No obstante, estos subtipos presentan gran diversidad agrupándose en linajes que se establecen en congruencia con factores temporales y geográficos, demostrado el impacto de estos en su historia evolutiva (S. Liu et al., 2009; Zhuang et al., 2019). Múltiples autores han propuesto diferentes nomenclaturas, sin embargo, el método estandarizado aceptado actualmente para nombrar los diferentes linajes de virus del subtipo H1 en cerdos es el propuesto por Anderson et al. (2016), que se basa en la filogenia y el origen de cada virus (Tabla 1-2). Respecto al subtipo H3, no existe un consenso, sin embargo, todos tienen un origen *human-like*, por lo cual Anderson et al. (2021) han propuesto la existencia de al menos cuatros linajes que son nombrados según la década de introducción a la población porcina, estos son *1970, 1990, 2000* y *2010*.

En la nomenclatura global de los virus del subtipo H1N1, el linaje clásico (1A) corresponde al primer virus introducido a porcinos en Norteamérica durante la pandemia de 1918 (Figura 1-9) (T. K. Anderson et al., 2021; Schultz-Cherry et al., 2013). Este linaje se relaciona con los descendientes del primer virus aislado y tiene un origen probable en salto inter-especie de humanos a cerdos (Anhlan et al., 2011; Shope, 1931; Taubenberger et al., 1997). El linaje Euroasiático (1C), de origen aviar, corresponde a los virus que emergieron en Bélgica en la década de 1970 (Figura 1-9) (Pensaert et al., 1981; Zell et al., 2013). En tanto que, el linaje *human-like* (1B), por su parte ha sido resultado de múltiples introducciones de virus estacionales humanos a poblaciones porcinas en diferentes partes del mundo (Figura 1-9) (M. I. Nelson et al., 2014; Rajao et al., 2019).

Tabla 1 - 2: Clasificación filogenética de los linajes del subtipo H1 en porcinos[10]

Linaje	Nombre	Sublinajes	Clados
1A	Clásico	1A.1 (H1-α)	1A.1.1 1A.1.2 1A.1.3
		1A.2 (H1-β)	-
		1A.3 (H1-γ)	1A.3.1 1A.3.2 (H1-γ-2) 1A.3.3.2 (H1N1pdm) 1A.3.3.3 (H1-γ)
1B	*Human-like*	1B.1	*European human-like*
		1B.2	1B.2.1 (H1-δ2) 1B.2.2 (H1-δ1)
1C	*Avian-like*	1C.1	-
	Euroasiático	1C.2	-

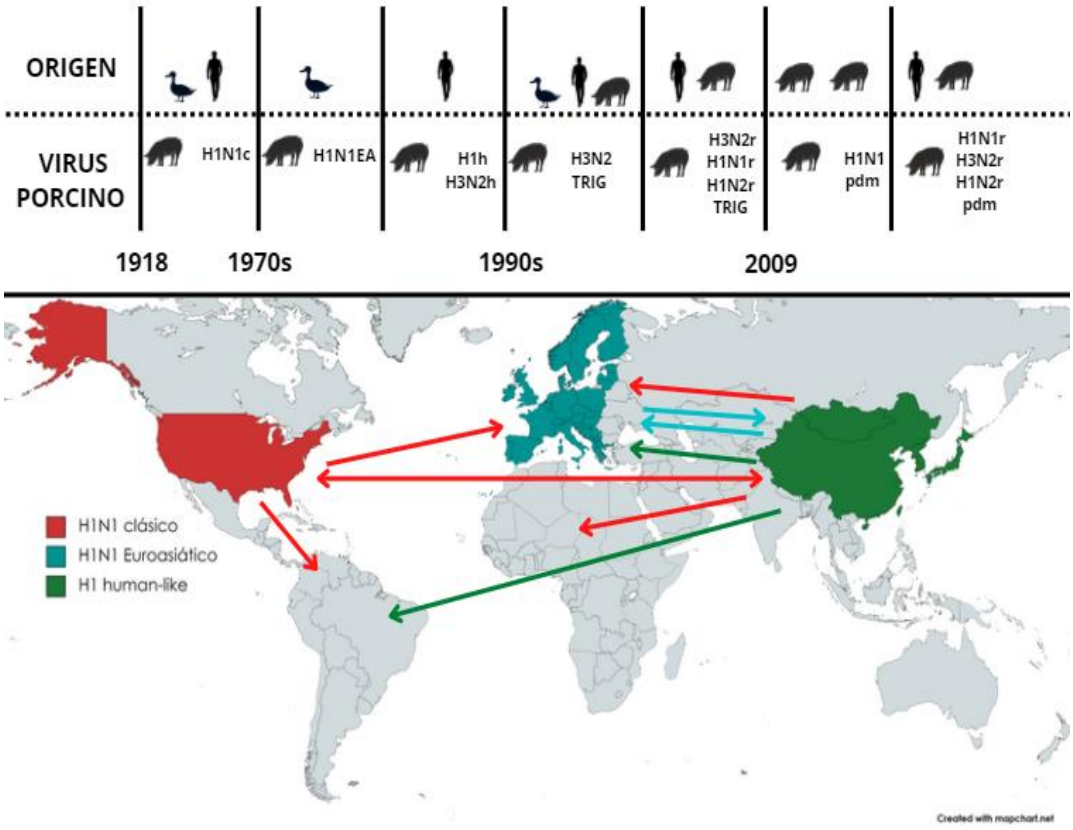
La continua circulación y coevolución de estos linajes propició el surgimiento de nuevas variantes por mecanismos de reordenamiento, incluyendo virus del subtipo H1N2, los portadores del set TRIG (*Triple Reassortant Internal Genes*) y el más reciente clado pandémico H1N1pdm09 (1A.3.3.2), que continúa circulando, adaptándose y reordenándose con virus endémicos, aumentando la variabilidad genética y antigénica del VIA (Figura 1-5) (Guo et al., 2020; Ma, 2020; Trebbien et al., 2013; A. L. Vincent et al., 2008; York & Donis, 2012; Zell et al., 2013).

En cada linaje, se han determinado diferentes clados, la mayoría de los cuales han demostrado distribuirse y emerger bajo patrones geográficos particulares. Dichos clados aparecen por cuenta de la segregación entre grupos de animales y el limitado tránsito interregional, que configura la existencia de nichos separados donde los virus circulan y evolucionan de forma independiente (Cheng et al., 2022). No obstante, el movimiento de linajes ha ocurrido en múltiples ocasiones (Figura 1-9), lo que ha llevado a la interacción, co-circulación y competencia entre diferentes variantes, resultando en fenómenos como el desplazamiento del linaje clásico y la dominancia del euroasiático en gran parte de Europa

[10] La clasificación más aceptada se basa en codificaciones alfanumérica. En paréntesis se presenta la equivalente nomenclatura al sistema norteamericano. Adaptado de Anderson et al. (2016).

al igual que el desplazamiento del linaje clásico por el clado H1N1pdm09 (Bhatt et al., 2013; Joseph et al., 2018; Osorio-Zambrano et al., 2022).

Figura 1-9: Línea de tiempo de los principales linajes del VIA porcino y su movimiento geográfico[11]



[11] En el panel superior se presenta una línea de tiempo y especies de origen de los principales linajes del VIA en porcinos. Elaborado con base en: Ma, (2020). En el panel inferior se presentan los movimientos geográficos de los linajes de H1; H1N1c: H1N1 clásico; H1N1EA: H1N1 Euroasiático; H1h: H1 *human-like*; H3N2h: H3N2 *human-like*; TRIG: portador del triple reassortant internal genes; H3N2, H1N1r, H1N2r TRIG: subtipos reordenados portadores de TRIG; H1N1pdm: H1N1 pandémico de 2009; H1N1r, H3N2r, H1N2r pdm: Subtipos reordenados portadores de genes internos del subtipo H1N1pdm. Elaborado en mapchart.com, adaptado de Cheng et al. (2022).

1.3.4 Características antigénicas del Virus de Influenza porcina

Al igual que en otras especies la inmunogenicidad del VIA en cerdos se centra en los mayores determinantes antigénicos HA y NA, por lo que los principios que dictaminan la antigenicidad de los VIA en porcinos corresponden a los descritos para los subtipos H1 y H3.

Para el subtipo H3, los cinco sitios antigénicos (A-E) son virtualmente iguales a los descritos en humanos, por lo que cualquier variación en estos se relación con *Drift* antigénico. Sin embargo, estudios hechos específicamente en la evolución antigénica del virus del subtipo H3 en cerdos indicaría la existencia de residuos específicos particularmente críticos (Tabla 1-3). En los H1, al igual en H3, los cinco mayores epítopes son los mismos, aunque existen ciertas posiciones que de igual forma sugieren ser prioritarias en el perfil antigénico del VIA (Tabla 1-3).

Tabla 1 - 3: Aminoácidos relacionados con el Drift antigénico en los principales subtipos de porcinos[12]

Subtipo	Aminoácidos*
H3	143, 145, 155, 156, 158, 159, 189
H1	142, 146, 147, 156, 158, 170, 171, 172, 180
N1	214, 22, 248, 273, 249, 273, 287,329, 349, 354, 451, 499
N2	199, 249, 254, 258, 263, 331, 332, 344, 346, 367, 369, 370, 400, 401, 402

[12] Elaborado con base en: Abente et al. (2016); Chastagner et al. (2020); Gao et al. (2019); Kaplan et al. (2021); Lewis et al. (2014); Luoh et al. (1992); Retamal et al. (2012); Sandbulte et al. (2011); Santos et al. (2019); F. Wang et al. (2021) y Ye et al., (2013). *El número del aminoácido puede variar según la numeración utilizada en cada estudio y las diferencias propias entre subtipos (Burke & Smith, 2014).

1.4 Diagnóstico del Virus de Influenza A en porcinos

Actualmente existen diferentes métodos para el diagnóstico de laboratorio del VIA en cerdos, no obstante, todos pueden ser clasificados según su principio en pruebas de tipo virológico, de biología molecular y serológicos. Tanto los métodos virológicos como los de biología molecular propenden por la detección del agente completo o de su material genético. En el caso del diagnóstico serológico, la mayoría de las pruebas buscan la detección de anticuerpos frente a proteínas virales que inducen una respuesta inmune como respuesta a la infección o vacunación, aunque también existen pruebas serológicas que buscan la detección de proteínas virales (ensayos de captura de antígeno) empleando anticuerpos. La relevancia de estas pruebas siempre dependerá del objetivo para el que se emplean (WOAH, 2023).

1.4.1 Pruebas virológicas

Las pruebas virológicas permiten la obtención y detección de partículas virales completas a través del aislamiento y el cultivo del virus utilizando sustratos biológicos. Los aislamientos deben ser confirmados con la técnica de Hemaglutinación (HA) que mide de forma indirecta la presencia de viriones y las pruebas de biología molecular que evidencian la presencia del virus a través de la detección del ARN viral. El aislamiento del VIA es un proceso complejo, laborioso y demorado (J. Zhang & Gauger, 2020). No obstante, es la prueba *gold* estándar para el estudio, monitoreo y entendimiento de las propiedades biológicas del VIA (FAO, 2010; WOAH, 2023). Las muestras empleadas para los aislamientos son tejido pulmonar, tráquea, hisopados nasales y fluidos orales (Brauer & Chen, 2015; Eisfeld et al., 2014; WOAH, 2023).

Actualmente, se reconocen dos principales metodologías para el cultivo y el aislamiento del VIA en cerdos. En primer lugar, se encuentra la utilización de huevos embrionados de

gallina, donde las membranas corioalantoidea y amniótica ofrecen un sustrato de replicación al expresar receptores tanto del tipo SAα2,3Gal como SAα2,6Ga (Sriwilaijaroen et al., 2009). Esta metodología fue inicialmente propuesta por Burnet (1936) quien describió la capacidad del virus de inducir lesiones en membranas embrionarias de huevos fértiles. Este sustrato ha sido empleado extensamente para el estudio del VIA y la producción de vacunas (Q. Chen et al., 2019; FAO, 2010; Rajaram et al., 2020) dadas las altas tasas de aislamiento que permite alcanzar (Clavijo et al., 2002). No obstante, presenta algunas limitaciones ya que se pueden inducir cambios adaptativos en las proteínas del virus, lo que afecta sus propiedades biológicas y genéticas (Gambaryan et al., 1998; Hardy et al., 1995; Stevens et al., 2010). Tales variaciones ocurren como respuesta a presiones de selección que se explican mayormente por desbalances en la expresión de las dos configuraciones del AS en las membranas embrionarias (Hardy et al., 1995; Sriwilaijaroen et al., 2009). El aislamiento del VIA por esta técnica se realiza a través de la inoculación de la cavidad alantoidea de huevos fértiles de entre 9 y 11 días que son incubados por 72 horas a 37°C (Brauer & Chen, 2015; Eisfeld et al., 2014; J. Zhang & Gauger, 2020).

En segundo lugar, se encuentra el aislamiento en cultivos celulares, el cual representa una alternativa viable al uso de huevos embrionados que además ofrece ventajas al proveer un sustrato de replicación altamente controlado y evitar la utilización de individuos vivos (Dolskiy et al., 2020). Para esta técnica se emplean líneas celulares susceptibles como las MDCK (Madin-Darby canine kidney) o las CACO-2 (colorectal adenocarcinoma cells) (J. Zhang & Gauger, 2020). El aislamiento se lleva a cabo inoculando monocapas celulares incubadas por hasta 96 horas a 37°C en atmosferas suplementadas con 5% de CO2 (Eisfeld et al., 2014; J. Zhang & Gauger, 2020). A diferencia de lo que ocurre con el aislamiento en huevos, la replicación del VIA en cultivos celulares generalmente resulta en la obtención de virus más estables que exhiben un menor número de mutaciones (Barnard et al., 2021; Stevens et al., 2010).

1.4.2 Técnicas de biología molecular

Las técnicas de biología molecular son el principal tipo de pruebas utilizadas en el diagnóstico del VIA en poblaciones animales. Estas se basan en la detección del material genético del virus a través de reacciones de amplificación de ácidos nucleicos, siendo la técnica más empleada la Reacción en Cadena de la Polimerasa (PCR); aunque existen alternativas como la reacción de amplificación isotérmica mediada por *loops* (Asih et al., 2021). Dado que el VIA posee un genoma de ARN, para la implementación de pruebas de esta naturaleza previamente se requiere la síntesis de ADN complementarios (cADN) a partir de ARN viral a través de reacciones de Retrotranscripción o Transcripción Reversa (RT).

Actualmente organismos internacionales como la OMSA y la FAO han diseñado protocolos de referencia para la detección del VIA que exhiben una alta sensibilidad y especificidad. Estos protocolos establecen pruebas de RT-PCR en tiempo real (qRT-PCR) encaminadas a la detección de regiones conservada en el gen M o NP (FAO, 2010; WOAH, 2023). No obstante, las pruebas de RT-PCR convencional continúan siendo una alternativa viable. Otras pruebas moleculares que permiten la sub-tipificación mediante la detección de los genes HA y NA de los principales subtipos porcinos también han sido estandarizadas y desarrolladas (J. Zhang & Harmon, 2020).

Algunas de las ventajas de las pruebas moleculares en el diagnóstico del VIA en cerdos, es que pueden diseñarse metodologías para la detección de cualquier gen de este y otros virus respiratorios, por lo que diversos grupos han desarrollado protocolos con múltiples objetivos como la diferenciación de linajes o la detección simultánea de patógenos asociados al complejo respiratorio (Cong et al., 2022; Goto et al., 2023). Otra ventaja de estas técnicas es su acople a tecnologías de secuenciación que permiten la caracterización molecular de genes o incluso del genoma completo del virus. Actualmente unas de las pruebas moleculares más empleadas para la secuenciación del VIA es la RT-PCR multisegmento diseñada por Zhou et al. (2009) que permite la amplificación del genoma completo del VIA en una sola reacción.

Pese a que la RT-PCR tienen un buen desempeño diagnóstico, es necesario considerar que estas son afectadas por la variabilidad genética y mutaciones en el genoma del VIA (Hoang Vu Mai et al., 2019; Stellrecht, 2018a), situación que quedó demostrada con la emergencia del H1N1pdm09, el cual presentaba mutaciones en la región del gen de Matriz detectada por la RT-PCR estandarizada por la OMS, lo que requirió el ajuste de la técnica (Stellrecht, 2018b). Por esta razón, las pruebas de biología molecular requieren de constante evaluación para garantizar la adecuada detección del virus circulante en campo.

1.4.3 Diagnóstico serológico

El diagnóstico serológico del VIA en cerdos típicamente se utiliza para medir la exposición de los animales al virus o para determinar la respuesta inmune desencadenada por vacunación (WOAH, 2023). Teniendo en cuenta que en Colombia no está aprobado el uso de biológicos contra el VIA porcino, las herramientas serológicas son útiles para la vigilancia epidemiológica del virus, puesto que toda seropositividad es indicativa de infección natural. Actualmente las técnicas de serología recomendadas por la OMSA son el Inmuno-Ensayo Ligado a Enzimas (ELISA) y la Inhibición de la Hemaglutinación (HI), aunque protocolos de seroneutralización e Inhibición de la Neuraminidasa también se encuentran disponibles (Gao et al., 2016; Gauger & Vincent, 2020a, 2020b).

Actualmente los principales kits de ELISA comerciales son de tipo competitivo (o de bloqueo) y permiten la detección de anticuerpos de cualquier isotipo contra la NP (ej: IDEXX Swine Influenza Virus Ab Test). Algunos de estos kits han sido licenciados específicamente para uso en porcinos aún cuando su principio, basado en el bloqueo de antígenos de NP altamente conservados dentro del género *Alphainfluenzavirus*, permite que puedan ser utilizados en diferentes especies (Ciacci-Zanella et al., 2010). La ELISA de bloqueo tiene una alta sensibilidad y especificidad (91% y 97%, respectivamente) en la detección de anticuerpos contra el VIA en cerdos, incluso cuando son inducidos por virus de diferentes subtipos (Song et al., 2009). Sin embargo, los resultados de este tipo de pruebas no permiten determinar los subtipos y linajes circulantes. Por lo que para

determinar respuesta serológica frente a subtipos o variantes específicos es requerido el desarrollo de ELISAs para cada uno de ellos (Okumura et al., 2019). Anteriormente se desarrollaron kits de ELISA indirecta encaminados a la detección de anticuerpos específicos contra los principales subtipos porcinos. No obstante, múltiples estudios demostraron su baja utilidad como consecuencia de su sensibilidad a las variaciones antigénicas del virus (Barbé et al., 2009), siendo únicamente eficientes contra subtipos y variantes específicas que no necesariamente son las actuantes en campo (Skibbe et al., 2004; Yoon et al., 2004). Dichas pruebas dejaron de ser implementadas y salieron del mercado al no ser posible la inclusión de un antígeno universal representativo de cada variante dentro de los subtipos porcinos. Por esa razón, la OMSA recomienda el uso de ELISAs de bloqueo únicamente como prueba de tamizaje en la determinación de circulación viral y cálculo de prevalencias (WOAH, 2023).

En cuanto a la HI, esta prueba es considerada el *gold* estándar en el diagnóstico serológico del VIA y es la base de la caracterización antigénica del virus mediante técnicas de cartografía. La HI ofrece considerables ventajas frente a las pruebas de ELISA puesto que logra sensibilidades altas y detecta de manera temprana todos los isotipos de anticuerpos que además son subtipo-específicos (Yoon et al., 2004). Adicionalmente, permite establecer el estado inmunitario de los animales frente al virus dado que reconoce anticuerpos neutralizantes y los cuantifica (a mayores títulos mayor protección frente a la infección) (WOAH, 2023). Sin embargo, esta prueba es altamente susceptible a las variaciones antigénicas del virus, por lo que su efectividad es dependiente de la representatividad de los antígenos empleados durante su ejecución (Skibbe et al., 2004). Por tal razón, la OMSA dictamina que los antígenos implementados en este tipo de pruebas deben ser representativos del contexto local (WOAH, 2024)

1.5 Evolución y adaptación del Virus de Influenza A en cerdos

Como ha sido ampliamente demostrado, todos los VIA presentes en porcinos tienen un origen humano o aviar, no obstante, no toda introducción desde estas y otras fuentes

conlleva su establecimiento y adaptación en la especie, por lo que se requiere siempre de procesos evolutivos y de mejora de las características bajo un modelo evolutivo Darwiniano, donde los virus que se adaptan son los que se mantienen. En este contexto, el principal ambiente y mayor desencadenante de la selección evolutiva del VIA en cerdos son las células porcinas (Rajao et al., 2019).

1.5.1 Cambios en HA

Dado que la HA reconoce el AS a través del RBD, el cual consta de los residuos Y98, W153, H183 y Y195, los dominios del *loop* 130 (134-142), 150 (150-156) y 200 (220-230), y la hélice 190 (181-193) (Broszeit et al., 2019; Newhouse et al., 2009), los cambios en estas regiones son típicamente necesarios durante los procesos de adaptación del virus a nuevos huéspedes y al ambiente celular porcino.

Por tanto, los cambios en la especificidad del RBD en cerdos señalan la selección de mutaciones asociadas con el reconocimiento del AS de mayor abundancia en el tracto respiratorio porcino (Tabla 1-4). Tales cambios ocurren de manera paulatina tras el pasaje seriado del virus en células porcinas, donde se evidencia la emergencia de mutantes con diferentes características que tienden a dominar o desaparecer según su impacto en el *fitness* viral (Shichinohe et al., 2013), siendo mayormente seleccionadas las variantes afines a receptores de AS en conformación SAα2,6Gal (Bakre et al., 2020; L. M. Chen et al., 2011). Ejemplo de esto es el H9N2 aviar que, tras ciclos replicativos en cerdos, sesga su población hacia portadores de D225G, que presentan un tropismo por ambas configuraciones de AS, sugiriendo un paso intermedio hacia la selección de mutantes con mejor desempeño (Mancera Gracia et al., 2017). Actualmente se sabe que estos cambios ocurrieron durante la adaptación del subtipo H1 del linaje 1C, puesto que durante su evolución adquirió y modificó la HA en un proceso escalonado. Inicialmente, ganó afinidad por ambas configuraciones y una alta replicación, pero su transmisión era afectada, por lo que posteriores adaptaciones que fueron mantenidas involucraron la pérdida de tropismo

por receptores de SA en conformación SAα2,3Gal, a través de cambios N207Y y K311Q en el dominio HA_1 (Su et al., 2021).

Tabla 1 - 4: Principales mutaciones de la HA en subtipos porcinos y su impacto en la afinidad por receptores[13]

Gen	H1	Afinidad
Residuos críticos	**……190……225……**	
Original	……E……..……G……	SAα2,3Gal
Variantes en porcinos	……D……..……G……	Dual
	……E……..……D……	
	……D……..……E……	SAα2,6Gal
	……D……..……D……	
Gen	**H3**	**Afinidad**
Residuos críticos	**……226…228……**	
Original	……..……..Q……S……	SAα2,3Gal
Variantes en porcinos	……..……..Q……G……	Dual
	……..……..L……G……	SAα2,6Gal
	……..……..L……S……	
	……..……..V……S……	

Aunque muchos de los cambios que se presentan de forma experimental en el virus tras infectar tejidos porcinos no afectan directamente los residuos del RBD, muchos ocurren en sitios cercanos, por lo que pueden tener algún impacto en su conformación tridimensional. Otras mutaciones también presentes en esta glicoproteína llevan a la adquisición de mejor actividad de fusión a un menor umbral de pH en la región HA_2 haciendo al virus estable al pH del tracto respiratorio del huésped (Bourret et al., 2017; Mancera Gracia et al., 2017; Urbaniak et al., 2022; Yang et al., 2017).

Otro factor determinante en la evolución de la HA es la continua selección de variantes antigénicas que surgen por mutación o reordenamiento. Estos procesos alteran la secuencia de aminoácidos y afectan la estructura de la glicoproteína al igual que los

[13] En rojo la configuración predominante en porcinos. Elaborado con base en: Rajao et al. (2019) y Shichinohe et al. (2013).

patrones de glicosilación, lo que evita el reconocimiento y la neutralización por parte de anticuerpos (Lewis et al., 2016). Este proceso surge como consecuencia de la presión de selección inmunológica que ocurre de manera natural por la infección en los animales. Aunque el peso de tal selección es menor a la existente en humanos (Furuse et al., 2010), este ha sido suficiente para guiar la aparición de clústeres antigénicos en diferentes partes del mundo (Lewis et al., 2016). No obstante, es necesario considerar que la carga inmunológica en poblaciones porcinas se ha visto aumentada con la utilización de vacunas que estimulan la producción de anticuerpos contra la HA (Mclean et al., 1945; Parys et al., 2022; Platt et al., 2011) generando una mayor selección positiva y cuellos de botella evolutivos que ocasionan la persistencia de algunas mutaciones (López-Valiñas et al., 2021; Murcia et al., 2012). Es por esto por lo que se cree que existe relación de la inmunidad en los cerdos con las tasas de sustitución de nucleótidos en el gen de la HA (Diaz et al., 2013; Murcia et al., 2012). Estudios en animales con o sin inmunidad previa coinciden en que la mayor tasa de sustitución de nucleótidos ocurre en la HA, en la que hay selección positiva y se calculan valores de entre 7.6×10^{-3} y 2.94×10^{-4} sustituciones/sitio/año (Diaz et al., 2013; Ryt-Hansen et al., 2020). Análisis hechos por López-Valiñas et al. (2021) reportan que los animales vacunados presentan mayor variedad genética que los animales no vacunados, sugiriendo que la existencia de inmunidad previa es un factor que favorece al establecimiento de cambios mayores. Estos cambios son inducidos y mantenidos por anticuerpos que reconocen y eliminan algunas variantes seleccionando aquellas capaces de prevalecer (Kilbourne et al., 1983; Matsuzaki et al., 2014; C. Xu et al., 2022), un fenómeno que ha podido ser observado en otras proteínas como M2 (Zharikova et al., 2005).

La cantidad de cambios necesarios en los sitios antigénicos para la variación antigénica en la HA y NA es incierto. Por ejemplo, en H3 se ha demostrado que sustituciones simultáneas en los residuos 154 y 189, aumentan considerablemente la distancia antigénica, en tanto que una única sustitución en el residuo 158 confiere cambios antigénicos significativos en el subtipo H1 (Abente et al., 2016; Z. Wang et al., 2021). Globalmente, la acumulación de sustituciones en la HA ha llevado a que el VIA frecuentemente varíe sus características antigénicas y sus patrones de N-Glicosilación (Chang & Zaia, 2019; Schulze, 1997), lo que resulta en la existencia de grupos antigénicos

y variantes glicosiladas de HA (Hause et al., 2012; Lewis et al., 2016), al igual que distintas formas de la proteína (Deblanc et al., 2020).

1.5.2 Cambios en la polimerasa viral

La evolución de las proteínas constituyentes de la vARNpol ha sido mayormente investigada en PB2, en la cual se señala la mutación E627K como responsable de la capacidad adaptativa a células mamíferas. Esta marca es observada en aislamientos de virus aviares en porcinos (Wu et al., 2018), lo que indicaría la ganancia de mecanismos hacia una replicación más efectiva en mamíferos. Aún así, esta mutación no está presente en todos los virus de cerdos, incluso, según algunos análisis bioinformáticos, sustituciones en esta posición no son significativas o comunes durante la adaptación del virus (Karnbunchob et al., 2016). Sin embargo, vías evolutivas alternas han desarrollado variaciones compensatorias como D701N y Q591K pueden explicar la adaptación del VIA en cerdos (Bussey et al., 2011; Chan et al., 2016; Q. Liu et al., 2012; Urbaniak et al., 2022; Yang et al., 2017). Algunas otras sustituciones en aminoácidos en PB2 que parecen favorecer la actividad polimerasa, la transmisibilidad y virulencia del virus en porcinos son R447G, I554L, R340K, T271A, I478V, G590S y Q591R (Karnbunchob et al., 2016; Q. Liu et al., 2012; Urbaniak et al., 2022; Y. Zhang et al., 2012).

En PA se identifican efectos por cambios como T85I, G186S, L336M, E382D, E613K y S409N que favorecen la transmisión y actividad replicativa a temperaturas cercanas a las del tracto respiratorio porcino (Bussey et al., 2011; Karnbunchob et al., 2016; Urbaniak et al., 2022). De igual forma, la existencia de una forma truncada de PA-X ha sido seleccionada en la mayoría de los virus porcinos actuales, lo que sugiere su importancia en el *fitness* viral en esta especie (G. Xu et al., 2016).

En cuanto a PB1, se identifica la selección y persistencia de la variante Q621R en virus del subtipo H1N1, siendo un cambio que fue requerido durante la adaptación del linaje 1C (Karnbunchob et al., 2016; Su et al., 2021).

1.5.3 Cambios en otros segmentos

Cambios importantes observados en otros genes del VIA aparecen en los que codifican proteínas relacionadas con actividad de replicación, estabilidad y evasión de la respuesta inmune como los segmentos NP y NS, que han aumentado su contenido de uracilo a través del tiempo desde su introducción a la población porcina (Joseph et al., 2018). Estas tendencias se relacionan con mejor desempeño de los virus, por lo que permiten aumentar su patogenicidad, transmisibilidad y replicación, probablemente por cambios en la interacción entre proteínas y los ARN virales, al igual que el nivel de bloqueo de la respuesta antiviral (Joseph et al., 2018; Selman et al., 2012; Su et al., 2021; W. Zhu et al., 2019). En la NP se reconocen como variables los residuos 305, 313, 351 y 357, y en NS1 las posiciones 35, 38, 41, 55, 44, 81, 103, 106, 108, 125 y 189 (Evseev & Magor, 2021; Joseph et al., 2018; Selman et al., 2012; Su et al., 2021). Tras el pasaje seriado de VIA en porcinos, se observan cambios en otras regiones como NP-I63M, R132K y A156T, y NS1-E101K, M56I, V226I (Shichinohe et al., 2013; Su et al., 2021; Urbaniak et al., 2022) que, aunque no ocurren en los sitios de mayor variabilidad, se organizan en regiones cercanas.

Las variaciones antigénicas en NA ocurren en porcinos, aunque no de manera muy significativa y en menor frecuencia que en la HA (Diaz, Enomoto, et al., 2015). Lo que indica que la variación genética y antigénica en esta glicoproteína ocurre de manera independiente y en menor grado en relación con la HA (Sandbulte et al., 2011). Curiosamente, en la proteína NS1 existe también cierta selección inmunológica pese a no ser un determinante antigénico convencional (López-Valiñas et al., 2021).

1.6 Panorama actual del Virus de Influenza en cerdos

El panorama del VIA en porcinos es complejo puesto que múltiples factores interactúan en el contexto de las poblaciones animales. De los más reconocidos son los patrones geográficos, el movimiento de animales entre granjas y la presencia de hospederos susceptibles, al igual que la introducción de virus desde fuentes humanas y aviares, responsables de la circulación de virus genética y antigénicamente diversos (Cheng et al., 2022; Diaz, Marthaler, Corzo, et al., 2017; Diaz, Marthaler, Culhane, et al., 2017; Kaplan et al., 2021; Lewis et al., 2016; López-Valiñas et al., 2021; Ryt-Hansen et al., 2020). Esta diversidad en cada población porcina conduce a interacciones de dominancia, co-circulación, desplazamiento, reordenamiento y plasticidad genética entre los virus circulantes, en un proceso favorecido por las dinámicas propias de las producciones porcinas, donde coexisten huéspedes con niveles diferentes de inmunidad (Diaz, Marthaler, Corzo, et al., 2017; Diaz, Marthaler, Culhane, et al., 2017; Diaz, Perez, et al., 2015; López-Valiñas et al., 2021; Ma, 2020; Ryt-Hansen et al., 2020).

1.6.1 Europa

En Europa circulan los subtipos H1 y H3 de diversos linajes. En este continente, el H1 1A estuvo presente por poco tiempo y de forma limitada desapareciendo totalmente en 1993 al ser desplazado por el linaje 1C (Zell et al., 2013). En la actualidad, se han identificado virus H1 del linaje 1C y clados del linaje 1B diferentes a los detectados en Norteamérica (T. K. Anderson et al., 2016) al igual que diferentes virus H3 en diferentes combinaciones y genotipos. Estudios recientes indican la existencia de 31 genotipos diferentes y 12 combinaciones de HA y NA, siendo las principales la H1N1 1C, H1N1 1A.3.3.2, H1N2 1B, $H1_{1B}N1_{1C}$, $H1_{1c}N2$, $H1_{1C}N1_{1A.3.3.2}$, $H1_{1A.3.3.2}N2$, $H1_{1A.3.3.2}N1_{1C}$, $H3N1_{1C}$ y $H3N2_{1B}$ (Henritzi et al., 2020; Simon et al., 2014). Interesantemente, dada la estricta regulación en los países de la Unión Europea, la proporción de genotipos y constelaciones genéticas se han desarrollado de forma diferente en cada uno de los países miembros (Simon et al., 2014).

En general en esta región existe una marcada dominancia del subtipo H1N1 1C, seguido mayormente por el subtipo H1N2 1B, siendo el H3N2 el de menor prevalencia y el subtipo H1N1 1A.3.3.2 un subtipo circulante de proporciones generalmente bajas (Chastagner et al., 2020; Chepkwony et al., 2021; Henritzi et al., 2020; Hervé et al., 2019; Simon et al., 2014; Zell et al., 2020). Los patrones geográficos indican la ausencia del H1N1 1C en Reino Unido y Dinamarca, en donde la dominancia está dada por H1N1 1A.3.3.2 y un $H1_{1C}N2$, respetivamente, siendo este último un virus únicamente presente en Dinamarca denominado como $H1N2_{dk}$ (Henritzi et al., 2020; Ryt-Hansen et al., 2021). El subtipo H1 1A.3.3.2 también domina además en la región europea de Rusia (Danilenko et al., 2021). El subtipo H3N2 está presente en países del centro de Europa como Bélgica, Países Bajos, Italia y España, pero está ausente en Dinamarca y Reino Unido (Henritzi et al., 2020; Simon et al., 2014).

1.6.2 Norteamérica

Norteamérica también presenta una gran diversidad filogenética en los subtipos H1, H3, N1 y N2, que circulan en diferentes combinaciones y genotipos (Chauhan & Gordon, 2020, 2022; Ma, 2020). Todos tienen un origen humano y han evolucionado en al menos 18 grupos, 7 de H1, 8 de H3, 2 de N1 y 3 de N2. Del linaje 1A circulan 5 clados, el 1A.1, 1A.2, 1A.3.3.3, 1A.3.2 y 1A.3.3.2 (Rajao et al., 2018; USDA, 2020, 2021; Vincent et al., 2014). Del linaje 1B el clado 1B.2.2, con dos subgrupos 1B.2.2.1 y 1B.2.2.2, al igual que el 1B.2.1, todos estos evolucionaron a partir de la introducción de dos virus estacionales humanos en diferentes momentos (Rajao et al., 2018; USDA, 2020, 2021; Vincent et al., 2014). Del subtipo H3 circula el clúster IV (H3.1990.4) y los clados *H3.2010.1* y *H3.2010.2* (T. K. Anderson et al., 2021; Ma, 2020; M. I. Nelson, Vincent, et al., 2012; J. D. Powell et al., 2021; USDA, 2020, 2021). Respecto a NA, están presentes el linaje N1 clásico (N1 1A) y el clado N1 pandémico de 2009 (N1pdm), junto a tres N2 *human-like*, uno introducido en 1998 (N2/98), otro en 2002 (N2/02) y el más reciente en 2016 (N2/16) (Chauhan & Gordon, 2020; Kaplan et al., 2021; M. I. Nelson, Vincent, et al., 2012; J. D. Powell et al., 2021; Rajao et al., 2018; USDA, 2020; Walia et al., 2019). El programa de vigilancia de la USDA indica que para 2021, en Estados Unidos los virus dominantes eran del clado 1A.3.3.3 y del

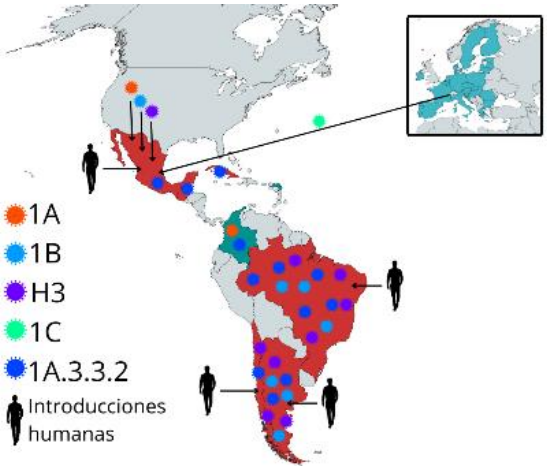
subtipo H3 del clúster IV-A (H3.1990.4), junto a N1 1A, N1pdm y N2/02, en combinaciones con genes internos TRIG/Mpdm/NPpdm, o TRIG/Mpdm, y su prevalencia varía en cada región (USDA, 2021; Walia et al., 2019).

1.6.3 Latinoamérica

De manera general en Latinoamérica no existe mucha información acerca de la diversidad genética y la dinámica evolutiva del VIA en cerdos. En México, la diversidad viral ha sido influenciada tanto por la introducción de virus humanos como por el movimiento de porcinos hacia su territorio desde diferentes regiones (Figura 1-10). Desde Norteamérica y Europa han sido introducidos virus del subtipo H1 del linaje 1A, 1B.2.1 (Mena et al., 2021; M. Nelson et al., 2019; Saavedra-Montañez et al., 2019), H3 del clúster VI (M. Nelson, Culhane, et al., 2015; M. Nelson et al., 2019; Saavedra-Montañez et al., 2019) y H1 1C (Figura 1-10) En Brasil, existen reportes de virus del linaje 1A, 1B, del subtipo H3N2 norteamericano y reordenamientos de H1N2 (Haach et al., 2019; M. Nelson, Schaefer, et al., 2015; Schmidt et al., 2014; Souza et al., 2019). Recientemente, también se evidenció la circulación de virus de los subtipos H3 (*1990.5*) y H1 (1B.2.3, 1B.2.4, and 1B.2.6) no contemporáneos que difieren genéticamente de los presentes en otras partes del mundo. Estos virus probablemente se establecieron tras saltos inter-especie de humano a cerdo ocurridos en ese país (Tochetto et al., 2023). En Chile, el panorama ha estado mayormente dominado por los subtipos H1N1pdm y H1N2, siendo detectada la circulación de dos clados divergentes del linaje 1B (chH1A y chH1B) que tienen un origen estacional humano (M. Nelson, Culhane, et al., 2015; Tapia et al., 2020, 2021). No obstante, el subtipo H3N2 también ha sido caracterizado y se ha confirmado su presencia en cerdos desde antes del 2009 al igual que la presencia de dos nuevos clados filogenéticos originados a partir de virus estacionales humanos (Mena et al., 2021; M. Nelson, Culhane, et al., 2015). En Argentina, en 2008 se demostró la presencia de un virus del subtipo H3N2 estacional humano completo (Cappuccio et al., 2011).

En resumen, a diferencia de lo observado en Europa y Norteamérica, la llegada del virus del clado 1A.3.3.2 a la región fue marcada por su dominancia frente a los virus endémicos. Tras su introducción a las poblaciones porcinas este ha sufrido reordenamientos aumentando considerablemente la variación genética (Dibárbora, 2016; Dibárbora et al., 2013; M. Nelson, Culhane, et al., 2015; M. Nelson et al., 2019; M. Nelson, Schaefer, et al., 2015; Osorio-Zambrano et al., 2022).

Figura 1-10: Panorama de la diversidad del VIA en Latinoamérica y Colombia[14]



1.6.4 Situación en Colombia

En Colombia existe una falta importante de información respecto a la diversidad y evolución de los VIA en porcinos. Análisis serológicos evidencian reactividad a los subtipos H1N1 1A y H3N2 desde finales de los 70 (Hanssen et al., 1977b; Mogollón et al., 2003; Moscoso Veloza & Neira Vásquez, 2001). Los primeros aislamientos y análisis filogenéticos se hicieron en 2008-2010, a partir de los cuales se caracterizaron molecularmente virus del linaje 1A y del clado 1A.3.3.2 (Ramirez-Nieto et al., 2012a). Posterior a esto y previo a este

[14] Elaborado en mapchart.com con base en: Dibárbora, (2016); Dibárbora et al. (2013); M. Nelson et al. (2015); M. I. Nelson et al. (2015), (2019) y Tapia et al. (2020).

trabajo, el linaje 1A no ha vuelto a ser reportado, mientras que el 1A.3.3.2 se ha identificado en diferentes regiones del país (Flórez Ramos et al., 2018; Karlsson et al., 2013; Mancipe Jiménez et al., 2014), y al menos hasta 2015 este ha permanecido siendo el virus dominante. Su evolución en el país indica que la presentación de *Drift* se ha dado en una forma dependiente de región, por lo que virus de los departamentos de Antioquia y Cundinamarca han dado lugar a subclados diferentes (Osorio-Zambrano et al., 2022). De igual forma, existe evidencia que sugiere la presentación de *Shift* entre virus H1 1A y 1A.3.3.2 poco tiempo después de la introducción del clado pandémico, no obstante, estas combinaciones no han prevalecido y no han sido reportadas nuevamente (Osorio-Zambrano et al., 2022). Finalmente, un reciente estudio encontró la circulación de genes internos de origen H1 1C previo a la emergencia del H1 1A.3.3.2 en Colombia, un hallazgo que sugiere que en el país pueden estar circulando genes y subtipos diversos que, ante la poca vigilancia y caracterización, han podido circular sin ser detectados (Gibbs et al., 2009; Osorio-Zambrano et al., 2022).

1.7 Conclusiones

El VIA es un patógeno complejo que representa un continuo reto para los sistemas de salud humano y animal. Al ser un virus que cambia y se adapta se hace necesario no solo conocer los factores involucrados en su evolución sino también mantener una vigilancia y monitoreo permanentes para evaluar las tendencias del *Drift* y la emergencia de nuevos virus y/o subtipos como resultado de procesos de *Shift*. La evolución de los subtipos del VIA en porcinos es un proceso complejo, multifactorial y poligénico, donde intervienen aspectos virales como su alta tasa de mutación y capacidad de reordenamiento. Sin embargo, su adaptación es guiada y las variantes genéticas son seleccionadas según su desempeño entendido como su capacidad infectiva, replicativa y de transmisión. Los factores del huésped impactan en este proceso al ser el microambiente donde los virus se desarrollan. En este sentido, los tejidos respiratorios porcinos ofrecen ventajas que permiten a VIA de distintos orígenes replicarse y adquirir mutaciones o segmentos genómicos que confieran características favorables para su adaptación al hospedero, en

un proceso que puede resultar en la emergencia de virus zoonóticos, dada la similitud que puede tener el tracto respiratorio porcino con el humano. Otro aspecto relevante lo constituye la presión de selección inmunológica que, al igual que en otras especies, en porcinos lleva a la emergencia de nuevas variantes antigénicas. Igualmente, es necesario considerar factores ambientales y de los sistemas de producción porcina, que pueden favorecer el mantenimiento y circulación del virus por la dinámica de la población animal, el efecto en la evolución del virus bajo patrones geográficos y la amenaza de introducción del virus desde y hacia los seres humanos.

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2. Sequence-Based Antigenic Analyses of H1 Swine Influenza A Viruses from Colombia (2008–2021) Reveals Temporal and Geographical Antigenic Variations[15]

2.1 Abstract

Swine influenza is a respiratory disease that affects the pork industry and is a public health threat. It is caused by type A influenza virus (FLUAV), which continuously undergoes genetic and antigenic variations. A large amount of information regarding FLUAV in pigs is available worldwide, but it is limited in Latin America. The HA sequences of H1 subtype FLUAV-positive samples obtained from pigs in Colombia between 2008–2021 were analyzed using sequence-based antigenic cartography and N-Glycosylation analyses. Of the 12 predicted global antigenic groups, Colombia contained five: four corresponding to pandemic strains and one to the classical swine H1N1 clade. Circulation of these clusters was observed in some regions during specific years. Ca2 was the immunodominant epitope among Colombian viruses. The counts of N-Glycosylation motifs were associated with the antigenic cluster ranging from three to five. The results show for the first time the existence of antigenic diversity of FLUAV in Colombia and highlight the impact of spatial and temporal factors on this diversity. This study provides information about FLUAV variability in pigs

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under natural conditions in the absence of vaccination and emphasizes the need for surveillance of its phylogenetic and antigenic characteristics.

Keywords: antigenic drift; bioinformatics; zoonotic virus; Alphainfluenza virus; Colombia.

2.2 Introduction

Swine influenza is a contagious respiratory disease of pigs that affects the pork industry globally and poses a continuous threat to public health. It is caused by the Alphainfluenzavirus (FLUAV) of the Orthomyxoviridae family [1]. FLUAV has a genome comprising of eight negative-sense RNA segments and is further classified into subtypes based on two major surface glycoproteins: Hemagglutinin (HA) and Neuraminidase (NA). At least 18 HA (H1–H18) and 11 NA (N1–N11) subtypes are recognized, all detected in wild aquatic bird species (Anseriformes and Charadriiformes), except for subtypes H17N10 and H18N11, identified in fruit bats in Guatemala and Peru, respectively; and H9N2-like FLUAVs, identified in fruit bats in Egypt and South Africa [2,3,4,5].

FLUAV has established permanent lineages in pigs worldwide. Established subtypes are H1N1, H1N2, and H3N2, each of which show significant phylogenetic and antigenic diversity, reflecting their ancestral origin and subsequent independent evolution [6,7,8,9]. Three H1 and four H3 swine lineages have been recognized, namely H1 1A (classical), 1B (human-like), and 1C (Euro-Asiatic or Avian-like) and H3 1970, 1990, 2000, and 2010-like strains [8,9].

Pigs are susceptible to FLUAVs of human and avian origin because of characteristics of their respiratory tract. These include the expression of receptors for human and avian origin FLUAVs (α2,6Gal and 2,3Gal, respectively) and supportive proteins needed for viral replication (swANP32A and swANP32B) [10,11,12]. Pigs are considered “mixing vessels” or intermediate hosts where FLUAVs from different origins can reassort, evolve, and potentially acquire mammalian adaptations or new genomic constellations, as observed during the emergence of the H1N1 pandemic virus in 2009 (clade 1A.3.3.2) [13].

FLUAV in swine undergoes continuous evolutionary changes owing to immunological selection pressures, resulting in antigenic drift [14,15,16]. These changes primarily occur in the globular region of the immunodominant glycoprotein HA, among which the epitopes and antigenic sites of both H1 and H3 have already been identified [17,18,19]. Even though selection pressure acting on swine populations is lower than that in humans [20], global studies indicate that it has been enough for the emergence of relevant antigenic diversity with numerous antigenic clusters reported in Europe, North America, and Asia [7,21,22]. The emergence of these new antigenic clusters is considered a potential risk to human health [21,22,23]. In Latin America, the antigenic characteristics and description of antigenic clusters of FLUAV in swine have only been performed in Chile, where different antigenic variants and new clusters were identified [24].

In Colombia, characterization of FLUAV in pigs has been limited to phylogenetic descriptions and serological surveillance reports. Epidemiological studies based on serology indicated that both H1N1 and H3N2 have been circulating among pig herds for at least 50 years [25]. More recently, research studies led to the isolation, sequencing, and partial genetic characterization of H1N1 strains in the country [26,27,28,29]. Regarding the H3N2 subtype, neither molecular evidence nor sequence data have yet been obtained. Consequently, knowledge about the virus is limited to the H1 subtype, specifically the 1A lineage represented by the classical 1A.1 (α-H1) and pandemic 1A.3.3.2 clades [26,27,29]. Evidence of these two clades suggests the existence of at least two antigenic clusters. However, it is possible that the virus has been undergoing antigenic drift, potentially leading to the emergence of antigenic variants and clusters that differ from those found in other countries, suggesting its independent antigenic evolution across the territory. This could be the result of different acting selective pressures and lack of vaccination against FLUAV in Colombia [26].

Therefore, this study aimed to evaluate the antigenic characteristics of swine H1 FLUAV in Colombia, estimate its antigenic variation, and describe potential antigenic clusters between 2008 and 2021. This study presents for the first time the results of antigenic characterization of swine H1 FLUAV in the country, which contributes to the understanding of the antigenic evolution of the virus under natural selective pressure in the absence of

vaccination. These results also highlight the importance of evaluating the antigenic characteristics of FLUAV in addition to phylogenetic analysis because of the underestimation of point mutations in antigenic regions.

2.3 Materials and Methods

2.3.1 Viruses

In this study, 37 full-length sequences of the HA gene of swine H1 FLUAVs belonging to the viral repository of the National Veterinary Diagnostic Laboratory of the Colombian Agricultural Institute (LNDV-ICA) and the Molecular and Virology Laboratory of the Universidad Nacional de Colombia (LBMV-UN) were used (Table 2-1). The viruses were obtained from nasal swabs and lung tissues of commercial pigs from high-density swine population regions of Colombia between 2008–2021 (Figure 2-1). Samples were collected during surveillance activities carried out by the LNDV-ICA and research projects conducted by the LBMV-UN. The procedures and conditions used to obtain samples from the LBMV-UN repository were approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science of the Universidad Nacional de Colombia.

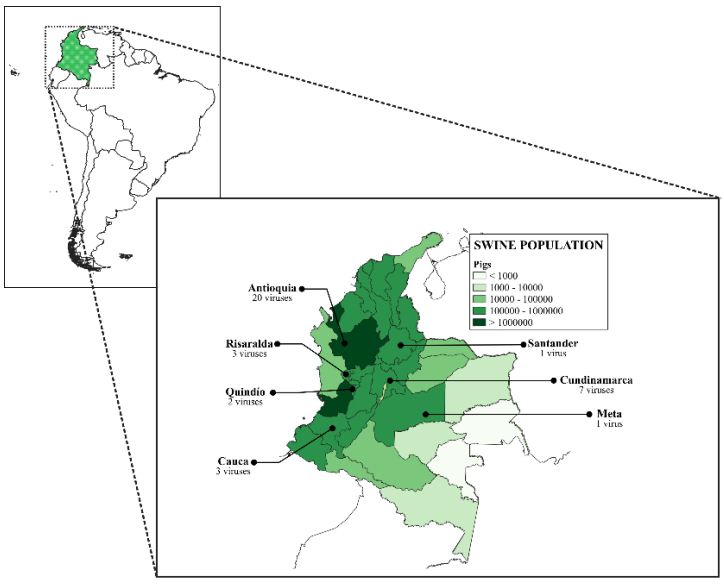
Table 2 - 1: Swine H1 Influenza A viruses included in the study.[16]

Virus ID	Year	Subtype	Lineage
16562/08/A	2008	H1N1	1A.1
16563/08/A	2008	H1N1	1A.1
16564/09/A	2009	H1N1	1A.1
16567/15/A	2015	H1N1	1A.3.3.2
16568/15/A	2015	H1N1	1A.3.3.2
14254/16/A	2016	H1N1	1A.3.3.2

[16] Virus ID corresponds to an internal number assigned by each laboratory plus the year and an acronym of the geographic region where the virus was detected. A: Antioquia, CA: Cauca, CU: Cundinamarca, M: Meta, Q: Quindío, R: Risaralda, and S: Santander. The lineage is presented in the global nomenclature.

14258/16/A	2016	H1N1	1A.3.3.2
14264/16/A	2016	H1N1	1A.3.3.2
14268/16/A	2016	H1N1	1A.3.3.2
14252/16/A	2016	H1N1	1A.3.3.2
14265/16/A	2016	H1N1	1A.3.3.2
14261/16/A	2016	H1N2	1A.3.3.2
14255/17/A	2017	H1N1	1A.3.3.2
08712/21/A	2021	H1N1	1A.1
08713/21/A	2021	H1N1	1A.3.3.2
14271/21/A	2021	H1N1	1A.3.3.2
08719/21/A	2021	H1N1	1A.3.3.2
08721/21/A	2021	H1N1	1A.3.3.2
14273/21/A	2021	H1N1	1A.3.3.2
14274/21/A	2021	H1N1	1A.3.3.2
14250/17/CA	2017	H1N1	1A.3.3.2
14256/17/CA	2017	H1N1	1A.3.3.2
14257/17/CA	2017	H1N1	1A.3.3.2
16565/10/CU	2010	H1N1	1A.3.3.2
16566/15/CU	2015	H1N1	1A.3.3.2
14253/16/CU	2016	H1N1	1A.3.3.2
08661/21/CU	2021	H1N1	1A.3.3.2
08663/21/CU	2021	H1N1	1A.3.3.2
14269/21/CU	2021	H1N1	1A.3.3.2
14270/21/CU	2021	H1N1	1A.3.3.2
14260/16/M	2016	H1N2	1A.3.3.2
14259/16/Q	2016	H1N1	1A.3.3.2
14251/16/Q	2016	H1N1	1A.3.3.2
14262/16/R	2016	H1N1	1A.3.3.2
14266/16/R	2016	H1N1	1A.3.3.2
14263/16/R	2016	H1N1	1A.3.3.2
14249/16/S	2016	H1N1	1A.3.3.2

Figure 2 - 1: Geographic origin of influenza A virus included in this study. All viruses were from regions with high swine population density.



2.3.2 Phylogenetic Characterization of the HA Glycoprotein

Nucleotide sequences of the HA gene of the viruses used in this study were previously acquired by next-generation sequencing (NGS) at The University of Georgia, USA. Used sequences were deposited in the GISAID database (Table 2-1). For the analysis, these sequences were translated into amino acids using the SeqBuilder ProTM Software v17.0 (DNASTAR Lasergene Inc, Madison, WI, USA). The protein sequences were aligned along with 61 H1 representative strains encompassing the three swine FLUAV lineages encompassing 1930 to 2021 and six seasonal human FLUAVs. Representative sequences were collected from the protein database of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/protein/; accessed on 13 January 2023) and are listed in Table S1. The alignment was performed with the MUSCLE-5 algorithm using the MUSCLE v5 tool [30]. The phylogenetic tree was constructed by the maximum-likelihood method using the ultrafast bootstrap approximation implemented in the IQ-TREE 1.6.12 software on a base of 1000 replicates [31,32]. The tree was edited using Interactive Tree of Life (iTOL; http://itol.embl.de; accessed on 10 February 2023) version 6.7.5.

2.3.3 Antigenic Characterization

Antigenic characterization was performed using the sequence-based antigenic cartography method developed by Anderson et al. [33] for H1 FLUAV. This method allows for the estimation of the antigenic distances (AD) between H1 viruses based on the amino acid differences in the five antigenic epitopes of the HA. For this analysis, protein sequences were edited and adjusted for HA1 numbering [34]. Subsequently, amino acids in the five major epitopes (Sa: 124, 125, 153–157, 159–164; Sb: 184–195; Ca1: 166–170, 203, 204, 205, 235–237; Ca2: 137–142, 221, 222; Cb: 70–75) were extracted using the Extractseq tool v6.6.0.0 from the European Molecular Biology Open Software Suite (EMBOSS) (https://www.bioinformatics.nl/cgi-bin/emboss/extractseq; accessed on 5 March 2023). Based on extracted peptides, an AD matrix was constructed by calculating and averaging the five epitopic distances (ED) between each virus using the cultevo v1.0.2 package in the RStudio® Software v4.3.1. The calculated ADs were represented in antigenic unities (AU), which are linearly correlated with the gold standard hemagglutination inhibition assay (HI) and suggest the existence of overlap recognition by antibodies at AD < 8.0 AU [33]. To infer antigenic clusters, a hierarchical clustering analysis was performed using the package stats4 v4.3.1 in the RStudio® Software. The same sequences used in the phylogenetic characterization were included in the antigenic cartography.

Antigenic maps were generated by applying a dimensional reduction to the AD matrix using the classical multi-dimensional scaling (MDS) of the stats4 package in the RStudio® Software. The optimal dimensional representation was chosen based on goodness-of-fit (GOF) calculations for dimensional spaces between 1 and 10, and the number of viruses to be represented in each map. Potential antigenic clusters were inferred based on the AD values observed between the classical and pandemic clades of the 1A lineage, and the clustering pattern observed in the hierarchical dendrograms. A K-value of 12 was selected to achieve higher discrimination resolution among both phylogenetic groups. The clusters were represented in a three-dimensional map, and the Colombian clusters in a two-dimensional map.

2.3.4 Epitope Analyses

The impact of each epitope on the antigenic clustering pattern was evaluated using median-joining network (MJN) analysis. For this purpose, amino acid consensus of the epitopes in each antigenic cluster was implemented. Consensuses were obtained using the Cons Tool from the EMBOSS v6.6.0.0 (https://www.ebi.ac.uk/Tools/msa/emboss_cons/; accessed on 8 March 2023). Networks for the epitopes were constructed using the NETWORK v10.2.0.0 tool (https://www.fluxus-engineering.com; accessed on 14 March 2023).

2.3.5 N-Glycosylation Analyses

The presence of N-glycosylation motifs (NxS/T; where x is any amino acid but P) in the sequences was evaluated using the NetNGlyc-v1.0 tool from the Technical University of Denmark (DTU) (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0; accessed on 2 April 2023). In this analysis, the entire amino acid sequence of each HA (HA1 numbering) was used. Only motifs with an N-glycosylation potential > 0.5 were considered as potentially modifiable sites.

2.4 Results

2.4.1 Colombian Swine H1 FLUAVs of the 1A.1 Clade Remain Genetically Stable, Whereas the 1A.3.3.2 Clade Shows Phylogeographic Divergence.

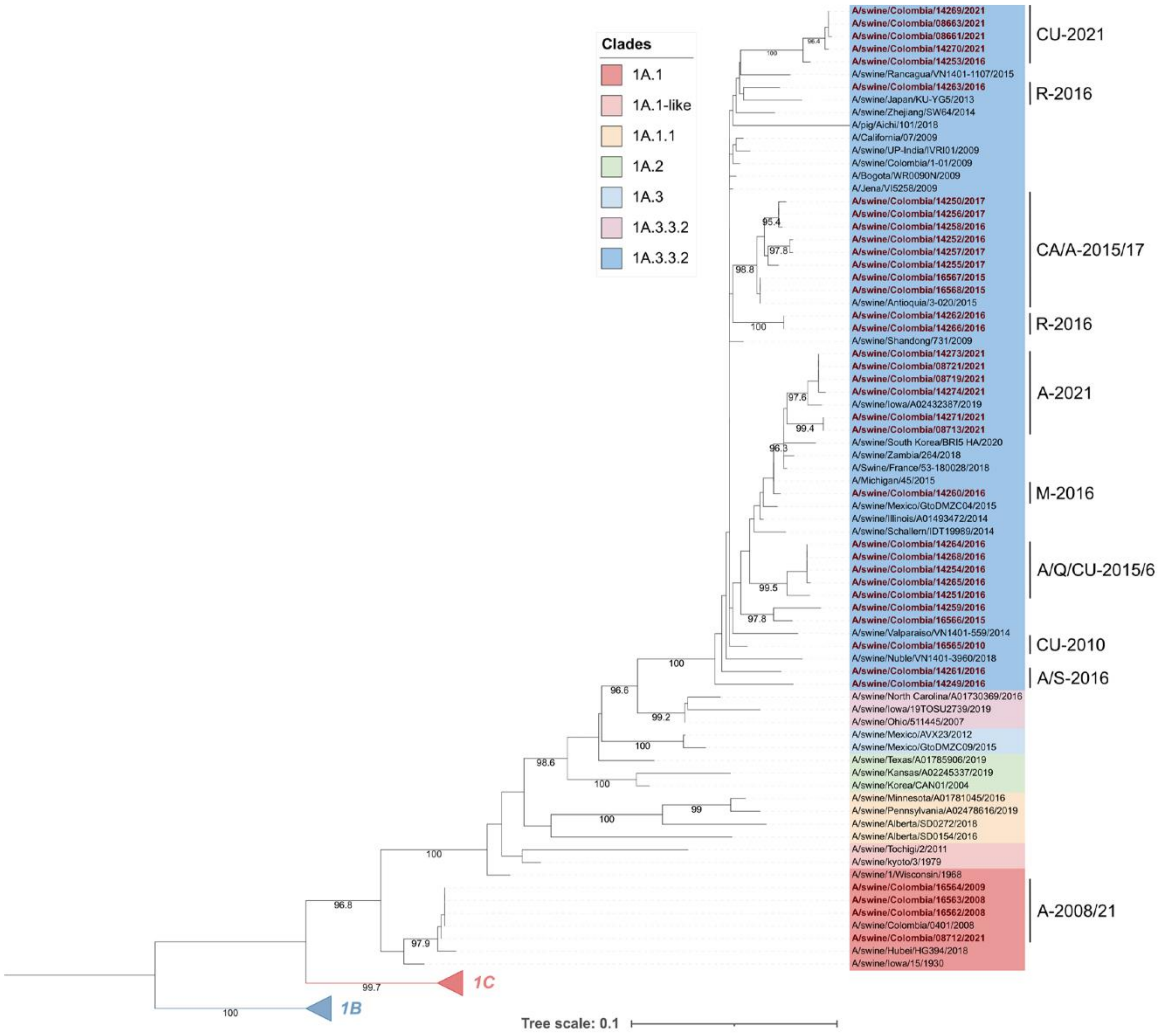
Phylogenetic analysis showed that Colombian viruses included in the study were grouped in the 1A lineage; 4 corresponded to the 1A.1 (α-H1) clade and 33 to the 1A.3.3.2 pandemic clade. The viruses in clade 1A.1 were placed into an early divergent branch into the lineage. These viruses were closely related to each other and constituted a monophyletic group, along with one strain from Asia (A/swine/Hubei/HG394/2018) and the ancient swine FLUAV A/swine/Iowa/15/1930 from North America (Figure 2-2).

Colombian viruses from clade 1A.3.3.2 had significant phylogenetic diversity. The FLUAVs from 2016 were the most diverse, as they were grouped into seven different subclades, one of which was a monophyletic group that differs from all the other viruses included in our analysis. On the other hand, viruses from 2015–2017 also displayed high phylogenetic diversity and were distributed intermixed into many subclades.

A regional trend in FLUAVs was observed since 2015, as strains from this year showed phylogenetic divergence according to their geographical origin. This tendency was also observed in the phylogenetic grouping of viruses in 2021 (Figure 2-2).

Figure 2 - 2: Phylogenetic tree of the HA glycoprotein of swine influenza A viruses included in this study.[17]

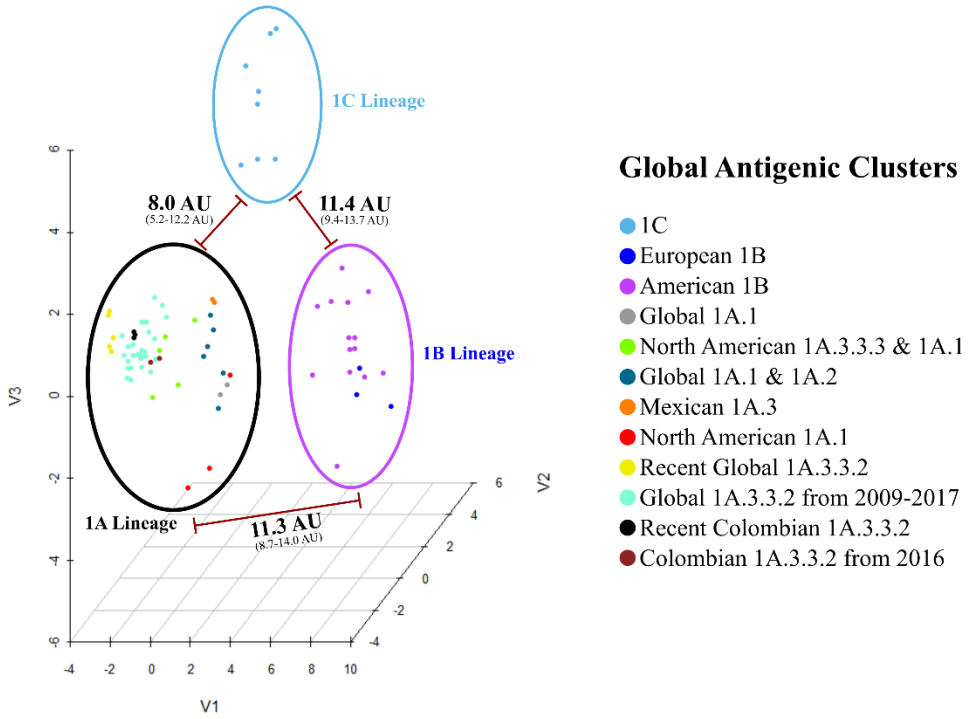
[17] Colombian viruses are labeled in red and highlighted in bold. Viruses were allocated into two clades corresponding to pandemic 1A.3.3.2 and classic 1A.1. Subclades where Colombian viruses were allocated are named according to the region and years of their detection, A: Antioquia; S: Santander; CU: Cundinamarca; Q: Quindío; M: Meta; R: Risaralda; CA: Cauca.



2.4.2 Sequence-Based Antigenic Cartography Shows the Relatedness with Phylogeny, Geographic Origin, and Temporal Factors

The sequence-based antigenic 3-D map showed three primary groups, each corresponding to one of the H1 Lineages. The 1A lineage exhibited the highest diversity comprising nine antigenic clusters, whereas lineages 1B and 1C were represented by two and one antigenic clusters, respectively. Each predicted cluster was named based on its phylogenetic, geographical, and temporal origin (Figure 2-3).

Figure 2 - 3: Sequence-based antigenic cartography of Colombian and global swine H1 influenza A virus.[18]

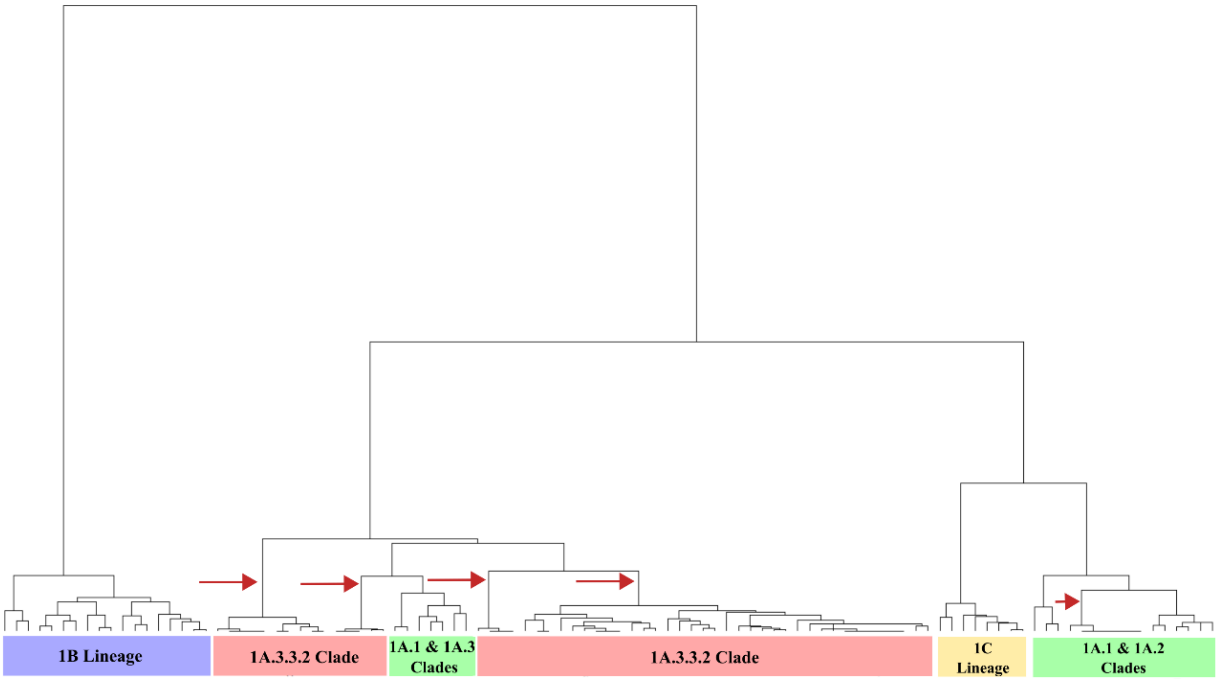


Among the sequences, the mean antigenic distance was 6.8 AU. The highest (14.0 AU) was found between recent Colombian isolates (08713/21/A and 14271/21/A) and the 1B European strain A/swine/Bakum/1832/2000. The lowest was 0.0 AU and was observed between FLUAVs with a similar geographic and/or temporal origin, as can be seen in the clustering pattern and the AD matrix (Figure S1). Hierarchical analysis showed that 1B Lineage was the most antigenically divergent group among the swine H1 FLUAVs, as it was positioned in a separated branch in the dendrogram and in a distant cluster in the antigenic maps. The 1A and 1C lineages were not strongly discriminated, and both were found to be mixed in the antigenic dendrogram. Colombian swine H1 FLUAVs included in

[18] The figure shows three major antigenic groups corresponding to swine H1 lineages. The mean calculated antigenic distances between groups are shown. Viruses analyzed are represented as points colored according to the assigned antigenic group.

this study were distributed across five branches that corresponded to five antigenic clusters, as shown in Figure 2-3 and Figure 2-4.

Figure 2 - 4: Antigenic dendrogram of the swine H1 FLUAVs analyzed.[19]



2.4.3 There Were at Least Five Antigenic Clusters Distributed in Different Regions of Colombia during Specific Years

The 1A lineage contained five antigenic clusters related to classical FLUAV and four to the pandemic 1A.3.3.2 clade (Figure 2-4 and Figure S2). The mean AD within lineage 1A was 4.3 AU (0.0–10.0 AU).

[19] Arrows show the branches in which Colombian isolates were allocated.

The classical clusters were Global 1A.1 (G1A.1), North American 1A.3.3.3 and 1A.1 (N1A.1–3), North American 1A.1 (N1A.1), Mexican 1A.3 (M1A.3), and Global 1A.1 and 1A.2 (G1A.1–2). G1A.1 was formed by viruses from the classical clade 1A.1 detected in the Americas between 1930 and 2021. In this group, the ancient strain A/swine/Iowa/17/19130, all classical Colombian viruses, and a virus from China detected in 2018 (A/swine/Hubei/HG394/2018) were allocated showing high antigenic stability (Figure 2-5). The N1A.1–3 group was diverse (Table 2-2) and included viruses from North America of the 1A.3.3.3 (γ-H1) and 1A.1 phylogenetic clades. The N1A.1 cluster comprised strains of the 1A.1 phylogenetic clade detected in the second half of the 2010s. This was the most divergent cluster, exhibiting an AD of approximately 8.0 AU, which suggests a lack of cross-reactivity with the others in the lineage. M1A.3 included two viruses of the γ-H1 phylogenetic clade identified in 2012 and 2015 in Mexico, notably (6.7 AU) distinct from other 1A.3 strains. The G1A.1–2 cluster contained strains of the 1A.1 and 1A.2 (β-H1) phylogenetic clades. These were subclustered according to a temporal pattern, with strains from the 2000s being grouped apart from the older viruses (Figure 2-5).

The pandemic clusters were Global 1A.3.3.2 from 2009–2017 (PDM09-17), Colombian 1A.3.3.2 from 2016 (PDM-CO-16), Global 1A.3.3.2 (PDM-21), and Recent Colombian 1A.3.3.2 (PDM-CO-21).

Figure 2 - 5: Antigenic dendrogram and clusters of the H1 1A lineage of the swine influenza A virus[20]

[20] Among the nine clusters, five corresponded to pandemic 1A3.3.2 and four to classic clades. Colombian viruses are highlighted in red.

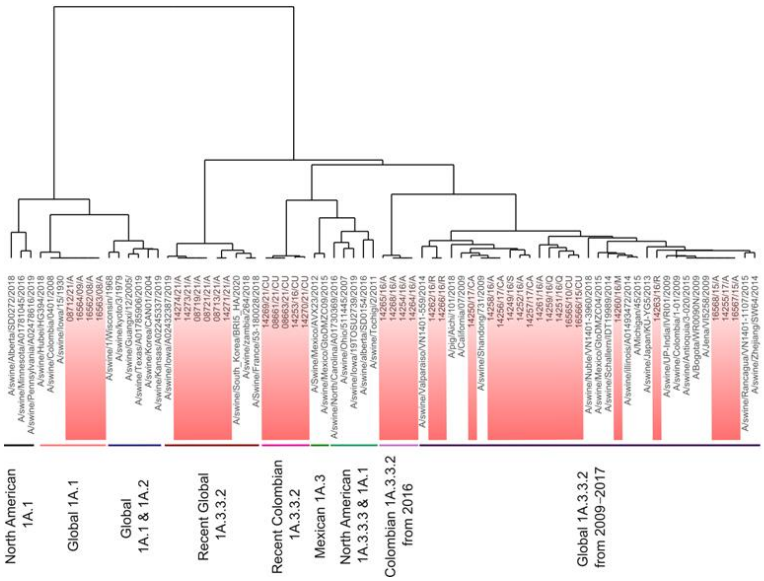


Table 2 - 2: Mean antigenic distance between predicted clusters of swine H1 1A lineage.[21]

	G1A.1	N1A.1	G1A.1–2	N1A.1–3	M1A.3	PDM-CO-16	PDM09-17	PDM-CO-21	PDM-21
G1A.1	0.3 *								
N1A.1	5.2	4.7 *							
G1A.1–2	2.8	6.8	2.6 *						
N1A.1–3	6.2	8.5	6.1	5.0*					
M1A.3	4.8	8.7	5.4	6.3	1.3 *				
PDM-CO-16	6.5	8.2	6.0	6.0	5.1	0.3 *			
PDM09-17	5.4	7.7	5.0	4.8	5.8	3.6	2.0 *		
PDM-CO-21	6.9	8.3	7.3	4.9	6.3	5.1	3.9	0.2 *	
PDM-21	7.2	8.1	6.8	5.8	7.3	4.5	3.1	3.5	0.9*

The PDM09-17 cluster had a mean AD of 2.0 AU (0.0–5.6 AU). It comprised three vaccine strains: A/California/07/2009 and A/Michigan/45/2015, used in humans; and A/Jena/VI5258/2009, used in swine. The human strain A/Bogota/WR0090N/2009 reported in Colombia was also grouped here, and it was antigenically similar to Colombian swine viruses detected between 2009 and 2017. A Chilean strain (A/swine/Nuble/VN1401-3960/2018) was the most divergent, with an AD of 3.0 AU from the nearest virus. In this

[21] * Mean AD within each cluster.

cluster, 18 Colombian viruses were identified. These had an AD of 2.1 AU (0.0–4.4 AU) among each other and were antigenically distant from viruses reported in Chile and Japan, but closer to viruses previously reported in Colombia, Europe, and Asia, as shown in Figure 2-5.

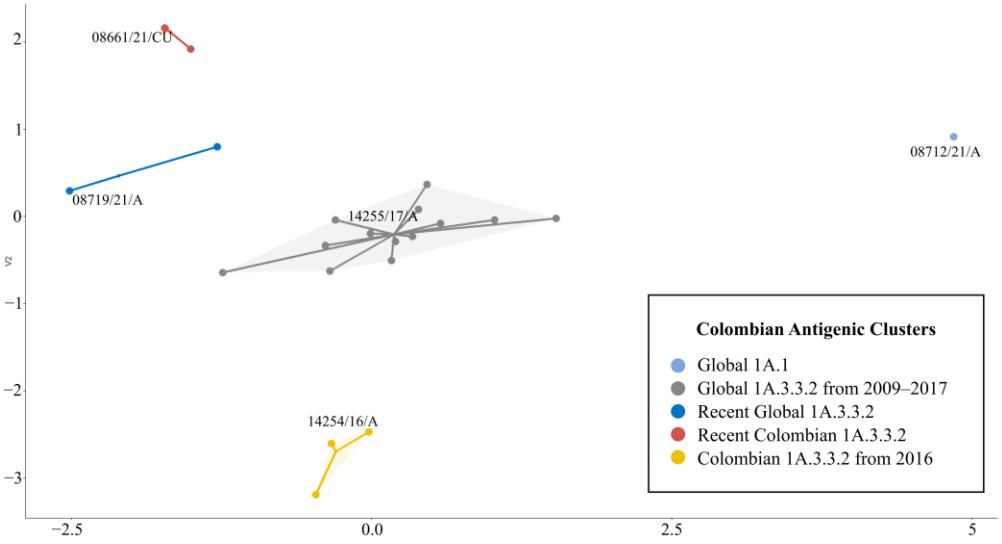
PDM-CO-16 cluster contained only four viruses from the Antioquia region identified in 2016. This group had a high antigenic relatedness. In the antigenic dendrograms and maps, this cluster was plotted near to the PDM09-17 (Figure 2-4, Figure 2-5 and Figure S2). However, based on the AD calculated between these two groups (3.7 AU), both were considered antigenically distinguishable. Nevertheless, a virus (14251/16/Q) from the PDM09-17 showed similarity to the PDM-CO-16 cluster by 1.7 AU.

The PDM-21 group comprised FLUAVs from North America, Asia, and Africa detected between 2018 and 2020, as well as six Colombian viruses identified in the Antioquia region in 2021. The cluster exhibited a high antigenic relatedness with a mean AD of only 0.9 AU (Table 2-2). Viruses detected during 2018–2019 were more antigenically related to each other than to those detected later during 2020–2021. Colombian viruses within the cluster were antigenically related to each other having an AD only of 0.8 AU (0.0–1.5 AU). In these viruses, the existence of a North American-like antigenicity was noticed in 14274/21/A, 14273/21/A, 08719/21/A, and 08721/21/A strains being only 0.3 AU apart from A/swine/Iowa/A02432387/2019. Conversely, a Eurasian-like antigenicity was observed in 08713/21/A and 14271/21/A, which were 0.8 AU apart from A/swine/Zambia/264/2018 and A/Swine/France/53-180028/2018.

In the PDM-CO-21 cluster, only five viruses were grouped. These were identified in the Cundinamarca region, four in 2021 and one in 2016. These FLUAVs displayed considerable antigenic similarity, with only an AD of 0.2 AU (0.0–0.3 AU).

Due to the low resolution of the AD between PDM09-17 and PDM-CO-16 clusters in the global three-dimensional antigenic map, a two-dimensional map containing only the Colombian isolates was constructed. In this cartography, the five antigenic clusters and their AD were clearly visualized, confirming that PDM-CO-16 is a divergent group located apart from other pandemic clusters. The 2-D map showed the PDM09-17 cluster as the central group, being located near all other clusters. In addition, it showed that recent PDM-21 and PDM-CO-21 clusters, despite being antigenically different from each other, have a similar tendency. The classical G1A.1 cluster was represented as a single point in a marginal position, proving their antigenic stability and divergence from the pandemic clusters (Figure 2-6).

Figure 2 - 6: Antigenic map of the Colombian H1 swine influenza A viruses.[22]



[22] Strains are represented as points colored interconnected according to the assigned antigenic cluster. In the groups, the names of representative strains are shown.

2.4.4 Antigenic Characteristics of 1B and 1C Lineages Were Partially Influenced by Phylogeny and Geographic Factors

Antigenic clusters of the 1B lineage were American 1B (1B-AM) and European 1B (1B-EU). The mean AD between both groups was 7.0 AU.

1B-AM had an AD of 4.1 AU (0.0–7.0 AU) and included viruses from Chile, Mexico, and the USA, as well as one strain from Vietnam and two human viruses (A/Medellin/WRAIR1297P/2008 and the vaccine strain A/Brisbane/59/2007). Viruses in this cluster tended to cluster according to regional and phylogenetic patterns (Figure S3). However, a high AD was observed for some phylogeographic-related viruses. This was the case of the Chilean viruses of the 1B.2-other clade and North American strains of 1B.2.2 (Table 2-3). In 1B-EU, the AD was 3.1 AU (0.0–5.6 AU) and the grouping pattern appeared to be related to the year of detection (Figure S3).

Table 2 - 3: Antigenic distance between influenza A viruses of the American 1B cluster according to their phylogenetic and geographic origins.[23]

	Chilean 1B.2-Other	**Mexican 1B.2-Other**	**Asian 1B.2-Other**	**North America 1B.2.1**	**North America 1B.2.2**	**Human**
Chilean 1B.2-other	3.1 *					
Mexican 1B.2-other	6.3	0.0 *				
Asian 1B.2-other	4.0	4.5	0.0 *			
North America 1B.2.1	4.2	5.7	3.8	2.9 *		
North America 1B.2.2	5.0	6.3	4.5	4.9	4.0 *	

[23] * Antigenic distance between members of the same geographic and phylogenetic origin.

Human	4.0	3.5	2.1	4.0	4.5	0.2 *

In the 1C lineage, the single antigenic cluster had a mean AD of 3.0 AU (0.3–6.3 AU). In this group, antigenic divergence was also affected by the geographic origin of the viruses, with larger distances between strains from different countries. This was found in all French viruses, where an AD > 4.0 AU from viruses from other countries was consistently calculated, except for A/swine/France/Cotes_dArmor-0388/2009. A three-dimensional map of the antigenic cartography of 1B and 1C viruses is shown in Figure S4.

2.4.5 Predicted Antigenic Clusters in Colombia Carried Point Mutations whitin the Epitopes, with Ca2 Demonstrating Immunodominance

MJN showed that the antigenic divergence among the 12 clusters was mainly determined by the average difference among the five epitopes. None of the networks displayed a single node for each antigenic cluster. Instead, networks contained between 9 and 11 nodes, indicating that some epitopes (Sa, Sb, and Cb) were similar in certain clusters, usually among viruses of the same lineage (Figure 2-7). Interestingly, the Sa epitope appeared to be conserved in strains of different lineages, as two clusters of the 1A lineage (PDM09-17 and G1A.1–2) were represented as a single node along with the 1C cluster. This conservation was also observed in the Ca1 epitope, in which all the 1A.3.3.2 clusters were identical. The Ca2 epitope had the highest inter-cluster resolution, representing 11 nodes, of which only 1 was shared and contained PDM-CO-21 and PDM-21 clusters. The network of this epitope also accurately reflected the phylogenetic origin of the FLUAVs (Figure 2-7). The MNJ of the five epitopes confirmed the antigenic divergence of the 1B lineage, with the two clusters always located apart from each other and from the 1A and 1C lineages.

Determination of point mutations in the epitopes of Colombian FLUAVs revealed no variations in classical viruses, which displayed 100% conservation. In contrast, pandemic

viruses exhibited variations, with the Ca2 epitope demonstrating immunodominance, as indicated by its low conservation percentage. Moreover, mutational tendencies related to the geographical origin and year of detection were observed (Table 2-4).

Figure 2 - 7: Median-joining networks of the five epitopes of swine H1 influenza A virus. Node size and color are related to the clusters and their phylogeny.[24]

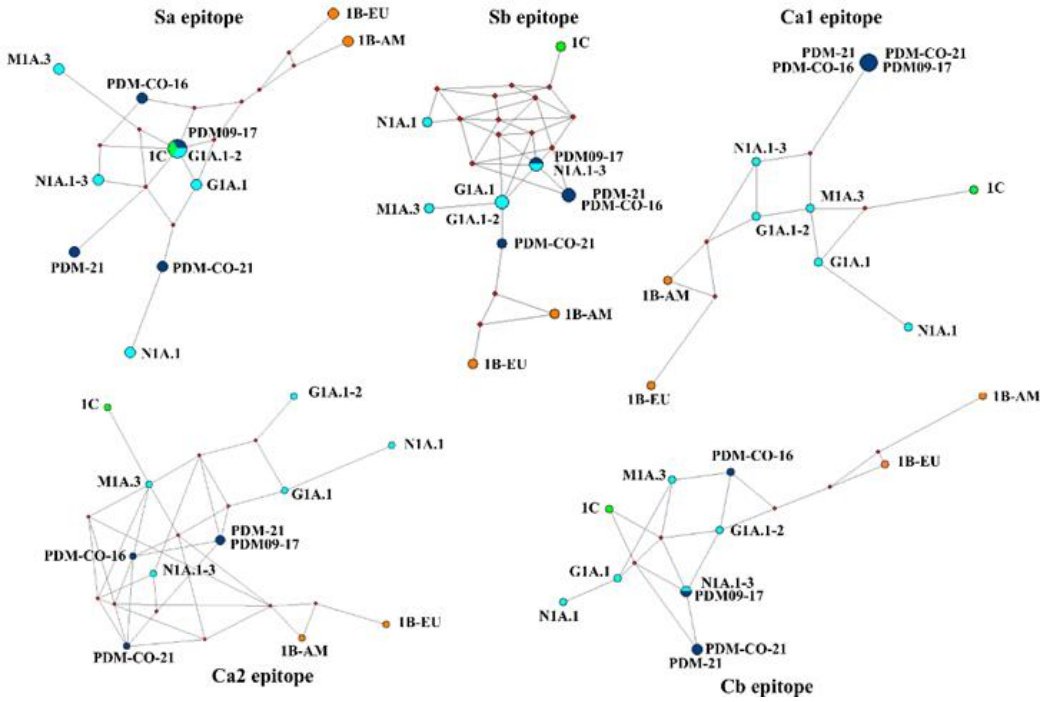


Table 2 - 4: Epitope conservation among Colombian H1 1A.3.3.2 swine influenza A virus.

Epitope	Conservation	Conserved Amino Acids	Mutations	Associated Region	Associated Year
Sa	38.5%	P124, K153, K154, S157, and P159	G155E	Cundinamarca	2021

[24] The phylogeny is represented with different colors: light blue for classical clades of the 1A lineage, dark blue for the pandemic clade of the 1A lineage, orange for the 1B lineage, and green for the 1C lineage.

			K160M	Antioquia	2021
			K160N	Antioquia	2016
			S162N	Antioquia Cundinamarca	2021
			S162Y	Antioquia	2021
			K163Q	Antioquia	2021
			K163I	Cundinamarca	2021
Sb	50%	D187, Q188, Q189, L191, Y192, and N194	A186T	Cundinamarca	2021
			S190H	Cundinamarca	2021
Ca1	63.3%	N167, K169, G170, S204, and G237	E235D	Antioquia	2021
Ca2	12.5%	G140	P137S	Cundinamarca	2021
				Antioquia	2016
			A141T	Cundinamarca	2021
Cb	50%	L70, T72, and G75	S71F	Antioquia	2016
			A73T	Antioquia	2016
			S74R	Antioquia Cundinamarca	2021

2.4.6 N-Glycosylation Motifs of Colombian Swine H1 FLUAVs Varied between Three and Five and Were Related to the Predicted Antigenic Cluster

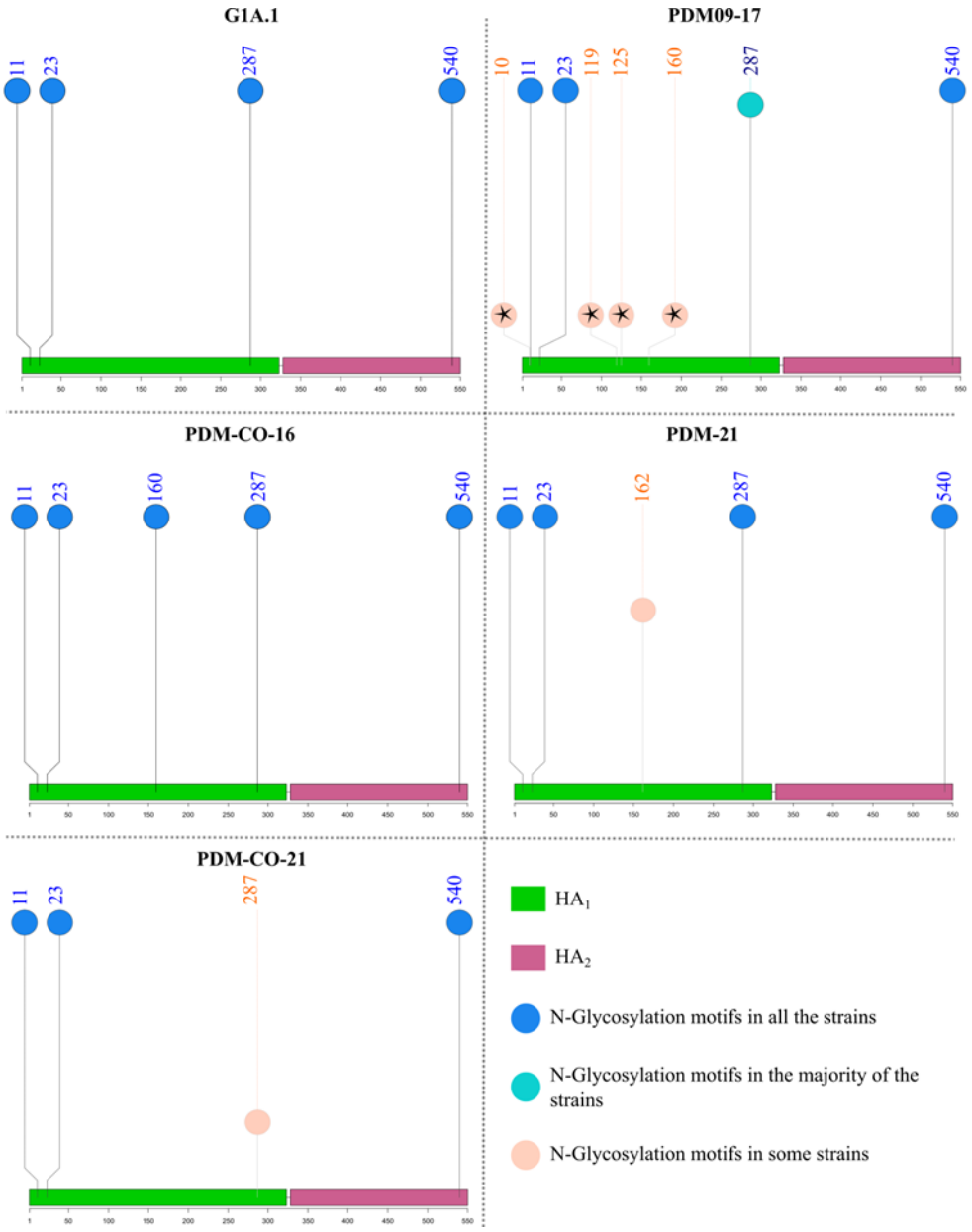
Motifs predicted in Colombian sequences are summarized in Table S2. Four consistent sites of N-glycosylation motifs were found across all H1 sequences at amino acid positions 11, 23, 287, and 540, except for the 1C lineage and some 1A.3.3.2 and 1B viruses. Among classical strains, only members of the N1A.1 cluster possessed five modifiable residues (consistent sites plus 162). In the G1A.1–2 cluster, A/swine/Kansas/A02245337/2019 contains an extra site at amino acid 10, making it a unique virus with five potential N-glycosylation sites. There was no gain or loss of N-glycosylation sites in classical Colombian viruses (Figure 2-8).

Regarding pandemic viruses, between three and six N-Glycosylation positions were observed, with the majority possessing the four consistent sites. Strains with three and six motifs belonged to the PDM09-17 cluster. These were A/Bogota/WR0090N/2009, in which the N-Glycosylation potential at 540 was lost, and A/swine/Valparaiso/VN1401-559/2014, in which additional positions were present at amino acids 10 and 119. Viruses with five sites were members of the PDM09-17 (A/swine/Nuble/VN1401-3960/2018 and A/swine/Indiana/A02524527/2020), PDM-21 (A/Swine/France/53-180028/2018,

A/swine/Zambia/264/2018, and all Colombian viruses), and PDM-CO-16 clusters. Colombian viruses from PDM-21 and PDM-CO-16 clusters had the additional motif at residues 162 and 160, respectively. Interestingly, in the PDM-CO-21 cluster, all viruses except 14253/16/CU lost one motif at position 287 (Figure 2-8).

Figure 2 - 8: N-Glycosylation motifs in the antigenic clusters of swine H1 influenza A virus where Colombian viruses were included.[25]

[25] The altitude of the circles represents the frequency of detection of the N-Glycosylation sites in each cluster. Motifs that were absent in Colombian viruses are marked with black stars.



All viruses in the 1C cluster had only three motifs at positions 11, 23, and 540, except for A/swine/Finistere/2899/1982, which had four motifs, including one at position 287.

The 1B lineage exhibited the highest motif counts, ranging from five to eight. Within 1B-EU, the four consistent sites, as well as one at 160, were displayed. The N-Glycosylation pattern

of the 1B-AM cluster revealed acquisition and loss of motifs according to the phylogeny of the viruses. Five motifs were observed in most strains of the 1B.2.1 phylogenetic clade; conversely, six were frequently detected in the 1B.2.2, located at positions 11, 23, 54, 125, 160, and 540. The 1B.2-other clade had the highest N-Glycosylation motif counts, with many strains possessing up to seven sites at 11, 23, 54, 125, 160, 287, 321, and 540. All the consistent sites were present in the human viruses, which displayed additional motifs at positions 54, 125, and 160.

2.5 Discussion

In this study, we provide for the first time in silico evidence of antigenic diversity among swine H1 FLUAV in Colombia using the sequence-based antigenic cartography approach. The method allowed for the inference of 12 global antigenic clusters, 5 of which were present in Colombia, with 2 detected only in the country. These results highlight the need for permanent surveillance of the antigenic evolution of the swine FLUAV in Latin America, particularly in countries like Colombia, where the virus might evolve unnoticed as could happened with the pandemic H1N1 virus after its introduction in 2009, increasing its zoonotic and pandemic risk.

The phylogenetic analysis of the Colombian viruses confirmed what has been previously proposed about the genetic and antigenic dominance of the pandemic clade over classical FLUAV in the country since 2008 with no indication of the introduction of new H1 lineages or phylogenetic clades [26,27,28,29]. Nevertheless, the results presented here also provide evidence of the maintenance of the classical virus in Colombian pigs until 2021.

In this research, 4.0 AU was suggested as a potential threshold for considering two swine H1 FLUAV strains as antigenically distinguishable by the in silico method. This value is proposed based on the calculated AD between classical and pandemic viruses, supported by evidence of significant antigenic variations and slight cross-reactivity between clades

[7]. Using this cut-off is supported by the findings of Anderson et al. [33] about the lack of overlapping antibodies recognition beyond an AD of 8.0 AU and the proved proportional loss of HI cross-reactivity between viruses when the highest ADs are calculated. However, it is important to note that this value must be validated through in vitro and in vivo approaches that captured the biological impact of specific mutations in the epitopes.

In Colombia, the five predicted antigenic clusters were related to the phylogeny of the viruses with one classical (G1A.1) and four pandemic (PDM09-17, PDM-CO-16, PDM-21, and PDM-CO-21) clusters. These were influenced by geographic and temporal factors.

The first antigenic cluster detected was the classical G1A.1, which was first identified in 2008 and persisted at least until 2021. This is likely the first established cluster in Colombia, considering that early serologic evidence suggests its circulation in the Antioquia region since the 1970s [25]. The virus was probably introduced from North America during the 1900s through the movement of live animals, as occurred in Asia [6,35]. This is supported by the phylogenetic relatedness of Colombian classical viruses with the ancient swine FLUAV strain reported in North America by Shope et al. in the 1930s [36] and an Asian strain. It is remarkable that despite its circulation for over 50 years in the country, the cluster has remained antigenically intact with no observed antigenic drift or posttranslational changes. Therefore, we propose that antigenic stability is the result of several factors. First, it is plausible that the low immunological pressure in Colombian herds, due to the absence of vaccination against FLUAV in pigs, has allowed for its circulation under no antigenic selective forces. Another factor is the population dynamics in swine herds, which allows for the persistence of naïve animals where the virus can replicate without significant immune pressure [37,38,39,40]. Finally, it is possible that the classical FLUAV has been circulating in Colombia at a low level under the shadow of the immunodominant pandemic clade.

After the emergence of the pandemic 1A.3.3.2 clade, the PDM09-17 cluster was established in the country, as happened in several countries around the globe. Once in the country, it remained the immunodominant group in the evaluated regions until 2021. This cluster was first introduced into the susceptible Colombian swine population during the

pandemic wave in 2009 [27], probably from human sources [41,42]. Because the introduction of the cluster occurred simultaneously in many geographic regions [42,43,44,45,46,47], the cluster contained viruses from Asia, Europe, and America. According to hierarchical analysis, some antigenic drift occurred, giving rise to two subclusters. This is consistent with previous reports on the diversification and antigenic variation of the pandemic HA during its dissemination [44,48,49,50], and accounted for the diversity observed at the intra-cluster level. Regarding the Colombian viruses within this cluster, the gain or loss of N-Glycosylation motifs were not detected. As a result, these possessed the same sites found in G1A.1, indicating relatively low immunological selection pressure among pigs in the country [51]. Concerning viruses from other countries, the gain of some N-Glycosylation motifs was noted in strains from Chile and India, probably due to their introduction into pigs after previous antigenic evolutionary steps in humans, as the gained sites have been related to the human host [51,52].

Interestingly, the pandemic PDM-CO-16 cluster cocirculated along with PDM09-17 only in the Antioquia region during 2016, and was not detected again. This group was both antigenically and phylogenetically distant from other pandemic viruses included in the study. The relationship of this cluster with 14251/16/Q within the PDM09-17 cluster suggests that PDM-CO-16 could emerge from strains of the earlier clade, and that Quindío's strain is an intermediate antigenic variant. It is also possible that this cluster was introduced into swine populations in Antioquia from humans, as we noticed an additional N-Glycosylation mark at position 160, which has been related to human adaptation [51,52]. In addition, we found that the cluster contained variations at the amino acid level at certain positions, and the existence of the mark P137S associated with the seasonal evolutive pattern of FLUAV in humans during the end of 2015–2016 in the Northern Hemisphere [53]. However, human-to-swine spillover events in Colombia are difficult to probe due to the absence of molecular surveillance of human FLUAV in the country.

Since 2021, two divergent pandemic clusters have appeared in the country: PDM-21 and PDM-CO-21. Both clusters were only detected in two geographically restricted swine

populations in that year, with PDM-21 limited to the Antioquia and PDM-CO-21 to the Cundinamarca regions.

Colombian viruses within PDM-21 showed antigenic profiles that were either American-like or Eurasian-like. The origin of this pattern could be related to the independent introduction of two genetically related FLUAV into pigs from different geographic sources during the international movement of animals and humans. It is probable that Colombian viruses from this cluster originated from a global human FLUAV, considering the presence of mutations K163G, E235D, and S74R, and the gain of an N-Glycosylation motif at 162 previously reported in pandemic viruses from humans [54,55,56,57]. The cluster likely reached swine populations in Eurasia and North America, from where it was then introduced to Colombia. It is possible to state this by considering the phylogenetic relatedness of Colombian strains of the cluster with FLUAVs detected in swine. Because of the observation that Eurasian-like viruses in the cluster (14271/21/A and 08713/21/A) did not have the S162N mutation that originated the additional N-Glycosylation motif, we propose that these viruses were introduced into pigs earlier than the North American-like ones.

The PDM-CO-21 cluster was entirely Colombian, and its first detection was performed in 2016. In 2021, it dominated the Cundinamarca region without evidence of antigenic drift. The apparent antigenic stability of the cluster indicates that once established in the swine population, it has been remained restricted to pigs [20]. This is supported by the high antigenic similarity between the recent isolates with the 14253/16/CU and their N-Glycosylation pattern, where recent isolates from 2021 have lost one motif at 287. This low level of posttranslational modification in HA has been associated with non-human hosts [51,58]. The origin of the cluster in the Cundinamarca region is uncertain; however, according to phylogenetic analysis, it was related to Asian, Colombian, and South American strains detected during the 2010s. This phylogenetic relatedness with FLUAVs detected in distant swine populations suggests its appearance during the frequent introduction of the pandemic clade in 2009 [41], or shortly after, during the diversification of the clade [48,49,50]. The absence of previous phylogenetic evidence of this cluster in the country

could be related to low molecular surveillance, which could have allowed for its circulation to go undetected, as has been proposed in the “unsampled pig herd theory” [59].

Regarding the predicted clusters of the 1B and 1C lineages, a high mean AD (>3.0 AU) was always observed, indicating low antigenic resolution of the method implemented in those groups. We believe this could be due to two main reasons. On the one hand, it is probable that the focus of our analysis for the 1A lineage affected the separative capacity of the sequence-based antigenic cartography in the 1B and 1C lineages. On the other hand, it is also possible that the number of clusters selected to represent antigenic diversity in the 1A lineage in this study (K = 12) was insufficient for the diversity in the 1B and 1C, and a higher K-value was required. These assumptions must be evaluated and validated in future studies.

In epitope analyses, the immunodominance among Colombian viruses was in Ca2, which is contrary to what has been reported previously in other countries where the epitopes Sa and Sb usually display major variation [60,61,62]. This could be a result of the low antigenic pressure among Colombian pigs due to the absence of vaccination that allowed for the conservation of epitopes located near the receptor-binding domain of HA. Intriguingly, antigenic conservation existed between global viruses at the Sa site of the 1A and 1C lineages. The relatedness of both groups has been previously observed using HI-based methods and is probably explained by their avian origin [7]. Considering that there is no evidence of a strong convergent evolution neither in 1A nor 1C [63], it is possible that the configuration of Sa has its origin in avian hosts, and once in swine, it has been maintained with minor changes due to a low selective pressure. However, it is necessary to consider that in the 1A lineage, the existence of an N-Glycosylation site at Sa could affect its antigenic similarity with the 1C lineage.

2.6 Conclusion

In this study we report, for the first time, evidence of antigenic drift and the existence of many potential antigenic clusters in swine H1 FLUAV in Colombia, contributing to the knowledge of antigenic evolution and diversity of the virus in Latin America and the rest of the world. The antigenic clusters found were influenced by spatial–temporal factors, as our results revealed the occurrence of independently new antigenic variants among Colombian regions. This study highlights the need for continuous surveillance and evaluation of not only the phylogenetic characteristics of swine FLUAV, but also its antigenic variations. This can be achieved by the sequence-based antigenic cartography method used here, as it represents a rapid, low-cost, low-labor, and useful tool for the study of antigenic characteristics and the selection of representative strains, which can be implemented in countries where the HI method is not an option or is not enough to provide full information about antigenic diversity. This information is essential to improve control and diagnostic strategies to minimize the health and economic impact of swine FLUAV in Colombia and the rest of the world.

2.7 Supplementary Materials

Table S1: Reference strains used in the phylogenetic and antigenic analyses.

Strain	Swine Lineage/Clade	Subtype	Country	Access number
A/swine/Iowa/15/1930	1A.1	H1N1	USA	ABV25634.1
A/swine/1/Wisconsin/1968	1A.1	H1N1	USA	ABV25636.1
A/swine/Guangxi/12/2005	1A.1	H1N1	China	ADV69039.1
A/swine/Hubei/HG394/2018	1A.1	H1N1	China	QOL21070.1
A/swine/Colombia/0401/2008	1A.1	H1N1	Colombia	AFU83122.1
A/swine/Alberta/SD0154/2016	1A.1.1	H1N1	Canada	ATB53861.1
A/swine/Minnesota/A01781045/2016	1A.1.1	H1N2	USA	AOX49803.1
A/swine/Pennsylvania/A02478616/2019	1A.1.1	H1N2	USA	QEO64518.1
A/swine/Alberta/SD0272/2018	1A.1.1	H1N2	Canada	QAX25196.1
A/swine/Kyoto/3/1979	1A.1-like	H1N1	Japan	BAG49742.1
A/swine/Tochigi/2/2011	1A.1-like	H1N2	Japan	BAM78376.1
A/swine/Korea/CAN01/2004	1A.2	H1N1	Korea	ACE77927.1
A/swine/Kansas/A02245337/2019	1A.2	H1N1	USA	QHF16399.1
A/swine/Texas/A01785906/2019	1A.2-like	H1N1	USA	QBC17636.1

a/swine/Mexico/AVX23/2012	1A.3.1	H1N1	Mexico	AMY15927.1
A/swine/Mexico/GtoDMZC09/2015	1A.3.1	H1N1	Mexico	AVI59052.1
A/swine/Valparaiso/VN1401-559/2014	1A.3.3.2	H1N1	Chile	AYV62774.1
A/swine/Rancagua/VN1401-1107/2015	1A.3.3.2	H1N1	Chile	ARV89702.1
A/California/07/2009	1A.3.3.2	H1N1	USA	YP_009118626.1
A/swine/Illinois/A01493472/2014	1A.3.3.2	H1N1	USA	AHY84443.1
A/swine/Mexico/GtoDMZC04/2015	1A.3.3.2	H1N2	Mexico	AVJ46821.1
A/Jena/VI5258/2009	1A.3.3.2	H1N1	Germany	AGA19175.1
A/swine/UP-India/IVRI01/2009	1A.3.3.2	H1N1	India	AIT38443.1
A/swine/Zambia/264/2018	1A.3.3.2	H1N1	Zambia	BDA36499.1
A/swine/Iowa/A02432387/2019	1A.3.3.2	H1N1	USA	QCQ05164.1
A/Swine/France/53-180028/2018	1A.3.3.2	H1N1	France	AZN23219.1
A/swine/Schallern/IDT19989/2014	1A.3.3.2	H1N1	Germany	ANA11539.1
A/swine/Nuble/VN1401-3960/2018	1A.3.3.2	H1N1	Chile	QDA17083.1
A/swine/South Korea/BRI5_HA/2020	1A.3.3.2	H1N1	Korea	QHW05456.1
A/swine/Zhejiang/SW64/2014	1A.3.3.2	H1N2	China	ALT19800.1
A/swine/Shandong/731/2009	1A.3.3.2	H1N1	China	AEF28990.1
A/pig/Aichi/101/2018	1A.3.3.2	H1N1	Japan	QPF21214.1
A/swine/Japan/KU-YG5/2013	1A.3.3.2	H1N1	Japan	AIT92741.1
A/swine/Colombia/1-01/2009	1A.3.3.2	H1N1	Colombia	AFU83120.1
A/Bogota/WR0090N/2009	1A.3.3.2	H1N1	Colombia	ACY77554.1
A/swine/Antioquia/3-020/2015	1A.3.3.2	H1N1	Colombia	AYD91054.1
A/swine/Ohio/511445/2007	1A.3.3.3	H1N1	USA	ACH69547.1
A/swine/North_Carolina/A01730369/2016	1A.3.3.3	H1N1	USA	AMM43240.1
A/swine/Iowa/19TOSU2739/2019	1A.3.3.3	H1N1	USA	QNH89409.1
A/swine/Scotland/410440/1994	1B.1	H1N2	Scotland	AAD05215.1
A/swine/Bakum/1832/2000	1B.1.2.1	H1N2	Germany	ACR39182.1
A/swine/Italy/60591/2018	1B.1.2.2	H1N2	Italia	AYM94710.1
A/swine/Illinois/A02139356/2018	1B.2.1	H1N2	USA	AVA07303.1
A/swine/Mexico/GtoDMZC01/2014	1B.2.1	H1N2	Mexico	AKQ43522.1
A/swine/Indiana/A02524527/2020	1B.2.1	H1N2	USA	QNL13973.1
A/swine/Alabama/A01104091/2016	1B.2.2.1	H1N2	USA	ANJ61799.1
A/swine/Illinois/A01644323/2018	1B.2.2.1	H1N2	USA	AUS83524.1
A/swine/Iowa/A02524587/2020	1B.2.2.1	H1N2	USA	QOH31678.1
A/swine/South Dakota/A02479076/2020	1B.2.2.2	H1N2	USA	QIP75475.1
A/swine/Chile/VN1401-274/2014	1B.2-other	H1N2	Chile	ARV89358.1
A/swine/Chile/VN1401-4/2014	1B.2-other	H1N1	Chile	ARV89416.1
A/swine/Chile/VN1401-339/2014	1B.2-other	H1N2	Chile	ARV89385.1
A/swine/Mexico/AVX61/2013	1B.2-other	H1N2	Mexico	AMY16231.1
A/swine/Binh Duong/02-16/2010	1B.2-other	H1N2	Vietnam	BAN14742.1
A/swine/O'Higgins/VN1401-4005/2018	1B.2-other	H1N2	Chile	QDA17400.1
A/swine/Finistere/2899/1982	1C.1	H1N1	France	AFR76505.1
A/swine/Belgium/1/1998	1C.2	H1N1	France	ACN67524.1
A/Swine/France/65-150242/2015	1C.2	H1N2	France	QJD66075.1
A/swine/France/22-200113/2020	1C.2	H1N2	France	QUQ73516.1
A/Swine/France/Cotes_d'Armor-0388/2009	1C.2.1	H1N1	France	AGK62667.1
A/swine/Italy/91162/2018	1C.2.1	H1N1	Italy	AYM94721.1
A/swine/Haseluenne/IDT2617/2003	1C.2.2	H1N1	Germany	ACR39185.1
A/swine/China/01/2019	1C.2.3	H1N1	China	QMS51333.1
A/Medellin/WRAIR1297P/2008	Human	H1N1	Colombia	AET84306.2
A/Michigan/45/2015	1A.3.3.2	H1N1	USA	QBL89789.1
A/Brisbane/59/2007	Human	H1N1	Australia	AJK02677.1

Table S2: N-Glycosylation motifs predicted in analyzed sequences.

Strain	Motifs	Residues
A/swine/Colombia/16562/2008	4	11, 23, 287, 540
A/swine/Colombia/16563/2008	4	11, 23, 287, 540
A/swine/Colombia/16564/2009	4	11, 23, 287, 540
A/swine/Colombia/16567/2015	4	11, 23, 287, 540
A/swine/Colombia/16568/2015	4	11, 23, 287, 540
A/swine/Colombia/14254/2016	5	11, 23, 160, 287, 540
A/swine/Colombia/14258/2016	4	11, 23, 287, 540
A/swine/Colombia/14264/2016	5	11, 23, 160, 287, 540
A/swine/Colombia/14268/2016	5	11, 23, 160, 287, 540
A/swine/Colombia/14252/2016	4	11, 23, 287, 540
A/swine/Colombia/14265/2016	5	11, 23, 160, 287, 540
A/swine/Colombia/14261/2016	4	11, 23, 287, 540
A/swine/Colombia/14255/2017	4	11, 23, 287, 540
A/swine/Colombia/08712/2021	4	11, 23, 287, 540
A/swine/Colombia/08713/2021	4	11, 23, 287, 540
A/swine/Colombia/14271/2021	4	11, 23, 287, 540
A/swine/Colombia/08719/2021	5	11, 23, 162, 287, 540
A/swine/Colombia/08721/2021	5	11, 23, 162, 287, 540
A/swine/Colombia/14273/2021	5	11, 23, 162, 287, 540
A/swine/Colombia/14274/2021	5	11, 23, 162, 287, 540
A/swine/Colombia/14250/2017	4	11, 23, 287, 540
A/swine/Colombia/14256/2017	4	11, 23, 287, 540
A/swine/Colombia/14257/2017	4	11, 23, 287, 540
A/swine/Colombia/16565/2010	4	11, 23, 287, 540
A/swine/Colombia/16566/2015	4	11, 23, 287, 540
A/swine/Colombia/14253/2016	4	11, 23, 287, 540
A/swine/Colombia/08661/2021	3	11, 23, 240
A/swine/Colombia/08663/2021	3	11, 23, 240
A/swine/Colombia/14269/2021	3	11, 23, 240
A/swine/Colombia/14270/2021	3	11, 23, 240
A/swine/Colombia/14260/2016	4	11, 23, 287, 540
A/swine/Colombia/14259/2016	4	11, 23, 287, 540
A/swine/Colombia/14251/2016	4	11, 23, 287, 540

A/swine/Colombia/14262/2016	4	11, 23, 287, 540
A/swine/Colombia/14266/2016	4	11, 23, 287, 540
A/swine/Colombia/14263/2016	4	11, 23, 287, 540
A/swine/Colombia/14249/2016	4	11, 23, 287, 540

Figure S1: Heatmap of the antigenic distance matrix among H1 swine influenza A Virus.

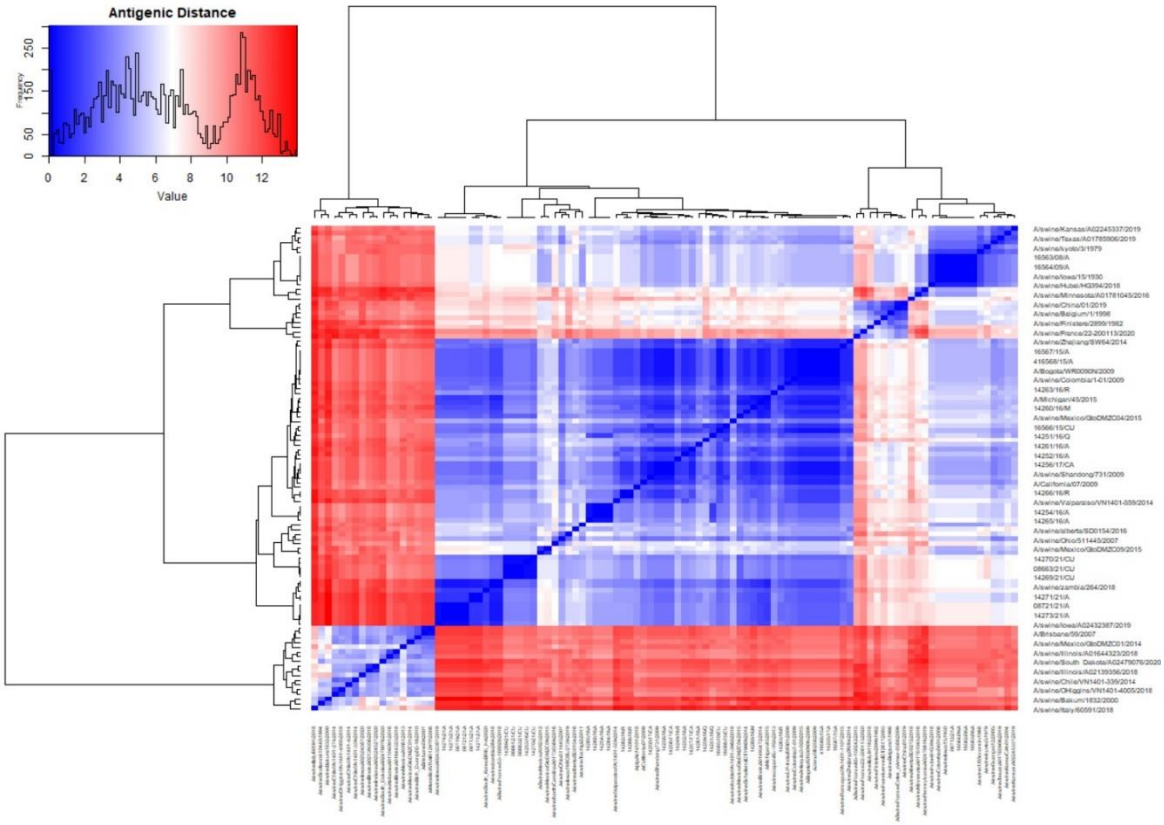


Figure S2: Antigenic three-dimensional map of the clusters in the 1A lineage.

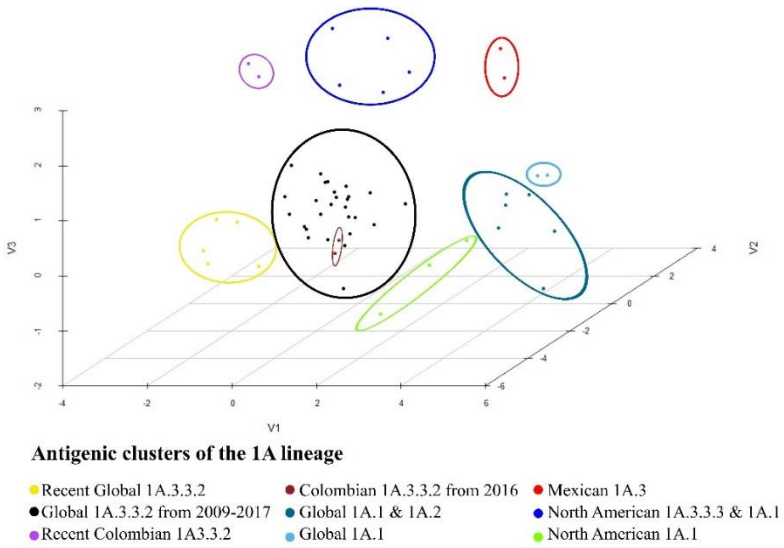


Figure S3: Antigenic dendrogram and clusters of the H1 1B and 1C lineages of the swine influenza A virus.

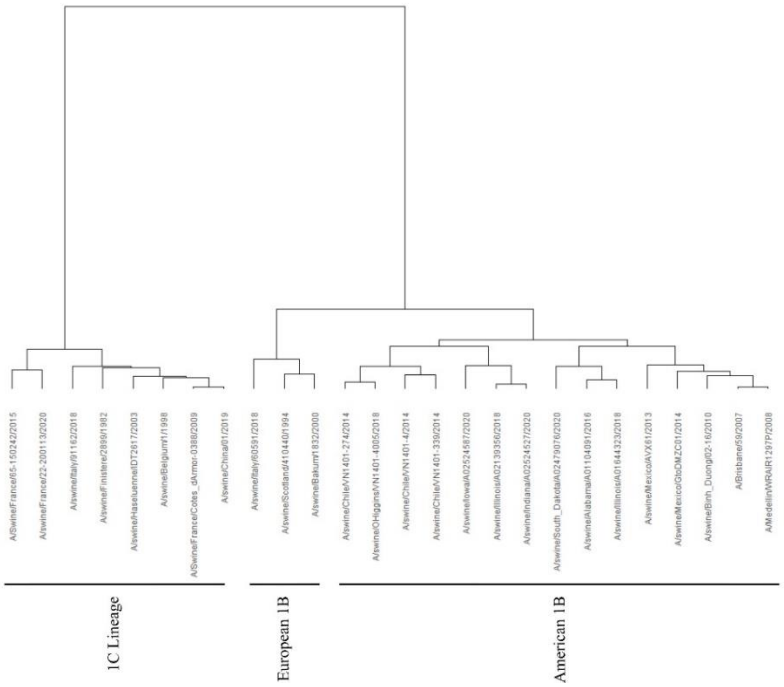
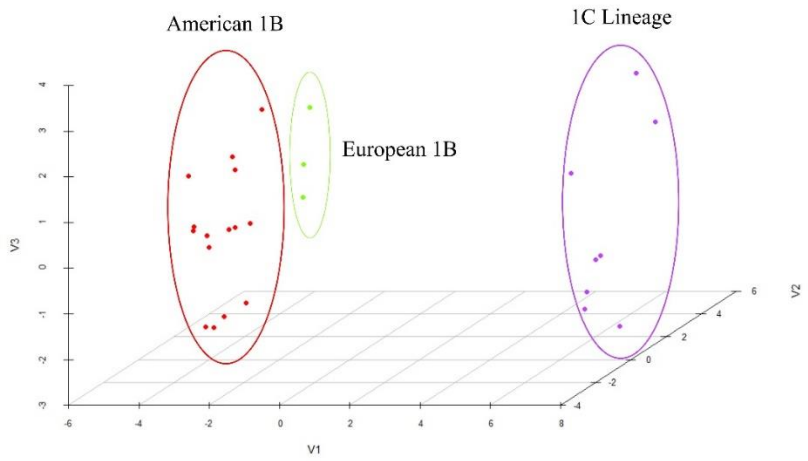


Figure S4: Antigenic three-dimensional map of the clusters in the 1B and 1C lineage.



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3. A Novel Reassorted Swine H3N2 influenza virus Demonstrates an Undetected Human-to-Swine Spillover in Latin America and Highlights Zoonotic Risks.[26]

3.1 Highlights

- First isolation and molecular evidence of the subtype H3N2 in swine in Colombia.
- New swine Influenza H3N2 virus discovered in pigs is phylogenetically divergent.
- Colombian H3N2 was originated from an independent human-to-swine spillover.
- Sequence-based analysis reveals that this is a novel antigenic variant.
- Due to antigenic variation, Colombian H3N2 possess a relevant zoonotic risk.

3.2 Abstract

Influenza A virus (FLUAV) affects a wide range of hosts, including humans and animals, representing a threat to public health. The origin of the H3N2 virus subtype in swine is associated with spillover of human seasonal viruses to pigs. However, the phylogenetic origin of swine H3N2 is poorly understood and the information about the molecular and

[26] Este capítulo será sometido para publicación en la revista *Veterinary Microbiology*.

antigenic characteristics of this subtype in Latin America is limited and completely absent in Colombia. Therefore, the objective of this study was to establish molecular characteristics of a swine H3N2 detected for the first time in Colombia. The origin and lineage of the virus were estimated through phylogenetic and molecular clock analyses. Antigenic characterization was achieved by comparing the amino acid constitution of the HA with previously reported swine FLUAVs and seasonal vaccine strains using a sequence-based method. Internal genes were also characterized. The Colombian H3N2 corresponded to a novel phylogenetic and antigenic swine FLUAV variant that emerged because of an independent reverse zoonotic event in the 2000s. It was found that the virus evolved through drift and shift, diverging from other human and swine FLUAVs contributing to the emergence of the novel Colombian swine H1N2 subtype. The immunodominant epitope in this FLUAV was mostly present in the antigenic epitope A. Some mutations that alter the N-Glycosylation of antigenic sites at the HA were detected. Internally, the virus had a pandemic configuration. This study provides the first evidence of a novel FLUAV in Colombia and describes its origin, variability, and persistence in a geographically restricted population, highlighting the need to enforce the characterization and molecular surveillance of the virus in animal populations.

3.3 Introduction

Influenza A Virus (FLUAV) is a globally distributed pathogen that affects a wide range of hosts, causing seasonal, epidemic, and pandemic outbreaks with devastating consequences for public and animal health. FLUAV has been related to the respiratory disease complex in pigs contributing to increase economic loses to the pork industry. The virus belongs to the *Alphainfluenzavirus* genus within the *Orthomyxoviridae* family and is classified into subtypes based on the combination of the two major surface glycoproteins, Hemagglutinin (HA) and Neuraminidase (NA), of which H1-H18 and N1-N11 subtypes have been recognized until now. The genome of FLUAV comprises eight single-stranded negative sense RNA segments which are prone to mutations and reassortment events. As

a result, the virus evolves continuously suffering changes at the genotypic and phylogenetic levels.

Currently three adapted subtypes of FLUAV are recognized in swine, these are the H1N1, H3N2, and certain reassorted H1N2, which display significant variation, forming phylogenetic lineages and clades. For H1 and N1, three lineages have been described and are designed according to their phylogenetic origin as classical (1A), *avian-like* (1C), and *human-like* (1B) (Anderson et al., 2016). Conversely, for H3 and N2, the lineages have been denoted considering their temporal origin as *H3-1970, -1990, -2000*, and *N2-1998, -2002*, and *-2016* (Anderson et al., 2021; Kaplan et al., 2021). Nevertheless, infections with non-adapted FLUAVs can occur due to unique characteristics of the swine respiratory tract. These infections could result in limited replication of the virus, dead-end infections and occasionally reassortment events with swine adapted FLUAVs that led to the emergence of novel genotypes that can persist in swine populations (Zhou et al., 1999). In addition, it is also possible that non-swine FLUAVs gain new characteristics through mutational changes allowing their adaptation to pigs as it was observed during the introduction of the 1C lineage in 1979 (Su et al., 2021). Consequently, pigs are recognized as “mixing vessels” and intermediate hosts where FLUAV can acquire new genomic characteristics that potentially enhance its zoonotic capacity and potential pandemic risk.

It is known that human-to-swine transmission of FLUAVs occurs frequently, resulting in the circulation of *human-like* genes in the swine viral pool (M. I. Nelson et al., 2014). The contribution of those genes to the appearance of new genotypes and antigenic variants with enhanced zoonotic capacity as well as their subsequent reintroduction into human populations is a matter of concern for public health (Yu et al., 2022). This thread acquires more relevance for the swine H3N2 subtype due to its entire human origin and similarity with previous circulating seasonal human FLUAVs (Cappuccio et al., 2011). Several studies have shown the frequent introduction of the H3N2 subtype from seasonal human FLUAVs in the swine viral pools in Europe, North America, and Asia (Brown, 2011; Kundin, 1970;

Zhou et al., 2000). Due to the limited information available, human-to-swine zoonotic movements of this subtype remain poorly understood in Latin America. However, recent studies have indicated independent introductions of seasonal H3N2 FLUAVs in Mexico, Chile, Brazil, and Argentina (Cappuccio et al., 2011; M. Nelson et al., 2015; Tochetto et al., 2023).

Nonetheless, the landscape of swine FLUAVs and human-to-swine spillover events is almost nonexistent in Colombia. Currently, the only known molecular data of the virus in swine came from the H1N1 subtype (Osorio-Zambrano et al., 2022; Ramirez-Nieto et al., 2012). Regarding the H3N2, neither molecular nor virus isolation have been reported yet, and the only evidence about the presence of this subtype in the country is limited to seroreactivity that indicates its circulation since the 1970s (Hanssen et al., 1977). Consequently, the molecular characteristics as well as the origin, genomic constellation, and antigenic properties of swine H3N2 in Colombia are unknown. Therefore, the objective of this study was to determine the phylogenetic and antigenic characteristics of the first swine H3N2 virus detected in Colombia. Results from this study contribute to the knowledge of swine FLUAV and shed light on the phylogenetic diversity of the virus in Colombia and the American continent. The study also highlights the need for a continuous molecular surveillance in developing countries where the introduction of new FLUAVs subtypes into swine can result in the undetected circulation, evolution, and emergence of novel strains with an increased zoonotic risk and pandemic potential.

3.4 Materials and Methods

3.4.1 Virus and genomic sequences

The swine H3N2 FLUAV analyzed in this study was obtained from a nasal swab sample collected during surveillance activities carried out by the National Veterinary Diagnostic Laboratory of the Colombian Agricultural Institute (ICA). The sample was taken from a commercial swine production located at Valle del Cauca region in 2017 and the isolation was achieved in SPF embryonated chicken eggs following standard procedures. The viral genome was fully sequenced by Next Generation Sequencing (NGS) at The University of Georgia, USA in 2021 and the sequences were provided in a FASTA format for the analysis. The virus was named A/swine/Colombia/14248/2017(H3N2) and the sequences of the whole genome were uploaded to the GISAID under the isolate identification EPI_ISL_18697751.

3.4.2 Phylogenetic analyses and molecular clock of HA and NA

HA and NA genes were first evaluated using the BLAST tool of the National Centre for Biotechnology Information - NCBI server on the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 18 November 2023). Lineages of both genes were determined through two approaches. The first was implementing the octoFLU pipeline, which allows the automated classification of genes according to their evolutionary origin based on their similarity with swine FLUAVs from North America (Chang et al., 2019). This approach was implemented in both HA and NA. For the HA, the second strategy consisted of an analysis that was carried out using the Subspecies Classification tool of the Bacterial and Viral Bioinformatics Resource Center (BV-BVRC) 3.30.5i (https://www.bv-brc.org, accessed on 18 November 2023). This methodology was considered confirmative of the lineage assignment of the HA. The second approach used for NA, was a BLAST analysis that allowed the comparison of the Colombian swine N2 with reference swine N2 genes published by Kaplan et al. (2021) available at https://github.com/flu-crew/n2-characterization.

Phylogenetic trees were constructed using the Maximum-Likelihood method with a 1,000 ultrafast bootstrap support in the IQ-TREE tool v1.6.12 (Hoang et al., 2018) after an alignment with data sets including representative human and swine FLUAVs using the MUSCLE-5 algorithm. For the construction of data sets, sequences for H3 and N2 genes were gathered from the GISAID (accessed on November 18, 2023) using the following criteria: Type A Influenza, H3 or N2 subtype, swine and human host, and complete HA or NA sequences only. Due to the large amount of genomic data for human FLUAVs, only viruses with whole genome sequences were considered for the construction of data sets for this host. After removing duplicates and strains with inexact data of collection, it remained 10,962 and 16,700 sequences of H3 and N2 respectively. From those sequences, representants were chosen using the CD-HIT-EST v4.8.1 tool considering an identity threshold of 98%. This resulted in final data sets that contained 432 H3 and 994 N2 sequences. Based on the topology of the phylogenetic trees and dates of collection, a molecular clock was performed with the TreeTime v0.11.1 program (Sagulenko et al., 2018). Time-scaled phylogenetic trees were visualized with the Interactive Tree Of Life version 6.7.5 (iTOL; http://itol.embl.de).

3.4.3 Phylogenetic analyses of internal genes

The origin and phylogeny of internal genes were inferred by the octFLU pipeline and the subsequent construction of phylogenetic trees under the same procedures used for the HA and NA genes. For those analyses, reference sequences for each genomic segment were collected from GISAID considering the following criteria: Type A Influenza, H1 or H3, N1 or N2, swine or human host, and fully sequenced genes only. Raw genomic data sets were reduced selecting representative sequences considering an identity threshold of 95% with the CD-HIT-EST tool. In the final data sets, unpublished sequences from swine H1N1 FLUAVs and from the novel strains of the H1N2 subtype from Colombia were included.

3.4.4 Antigenic characterization

The sequence-based *P-epitope* model was implemented for the antigenic characterization of the Colombian swine H3N2 (Gupta et al., 2006). This method allows the calculation and visualization of antigenic distances between FLUAVs based on amino acid differences in the five canonical epitopes of the HA (namely A, B, C, D, an E). It also predicts the efficacy of human vaccines against wild strains allowing an estimation of zoonotic risk. According to the model, when a *P-epitope* value >0.22 between the vaccine and circulating strains exists, the vaccine-induced immune response has a negative impact due to antigenic mismatch.

For this analysis, the amino acid sequence coded by the HA gene of the Colombian swine H3 was inferred using the orfipy v0.0.4 tool. This predicted protein was aligned with 21 amino acid sequences downloaded from the protein database of the NCBI (https://www.ncbi.nlm.nih.gov/protein, accessed on 17 December 2023), that corresponded to human seasonal vaccine strains used between 1968 and 2023; and 27 proteins from swine FLUAVs that were selected taking into consideration strains from the genomic data set constructed for the HA phylogenetic analysis. The 27 selected swine proteins were chosen with the CD-HIT tool considering an amino acid identity of 90%. Both human and swine viruses used for this analysis are listed in Table S1.

For the implementation of the model, amino acids in the epitopes were pulled out from the alignment and compared using the *stringdist* and *stats4* packages in the RStudio® software (R 4.3.1). To obtain the P-epitope values, a fractional difference (*Pk*) matrix for each epitope was first constructed applying the formula reported by Bonomo et al. (2019). Then, *Pk* values were compared to construct a matrix containing the *P-epitope* values (the greatest *Pk* value). The *P-epitope-distances* were visualized in two dimensional maps after a Multidimensional scaling (MDS) as reported previously (Bonomo et al., 2019). As an

indication of zoonotic risk, the efficacies and protection of vaccine strains used in 2016-2018 and 2018-2019 against the Colombian swine H3N2 were calculated (Bonomo et al., 2019; Bonomo & Deem, 2018). In addition, the presence of marks associated to antigenic drift, N-Glycosylation, receptor tropism, and antiviral resistance in the HA and NA genes were assessed using the FLUserver tool v1 available at GISAID.

3.5 Results

3.5.1 The Colombian H3N2 swine Influenza virus was originated from a novel independent human-to-swine spillover that took place in the early 2000s.

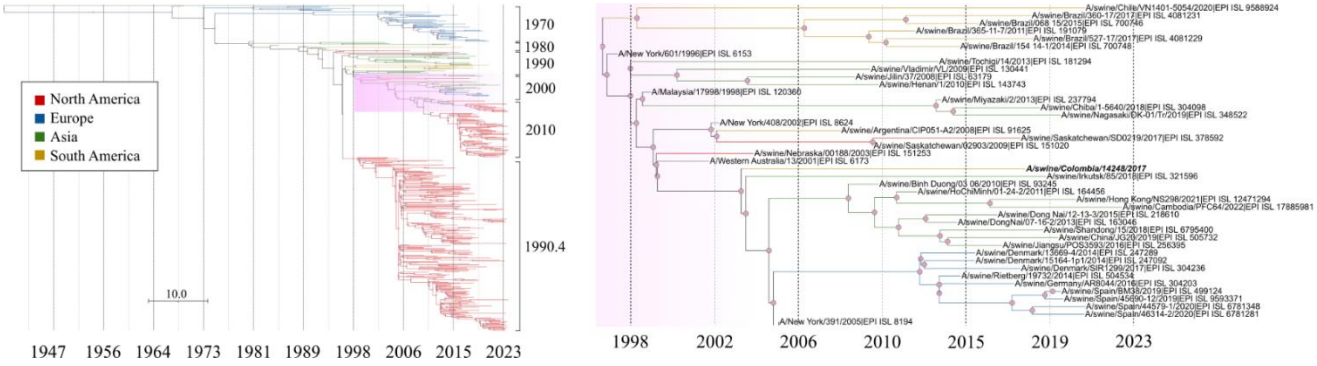
The BLAST analysis indicated that both HA and NA genes of Colombian swine H3N2 were related to seasonal human FLUAVs that circulated in North America in 2003. Nevertheless, the most similar strains exhibited a low nucleotide identity in both genes (94.10% in HA and 95.70% in NA). According to the octoFLU assignment, A/swine/Colombia/14248/2017(H3N2) had an HA and NA from the *H3-2000* and *N2-1998* lineages. However, it showed a low similarity with North American FLUAVs, being weakly associated to the A/swine/Texas/4199-2/1998(H3N2) reference strain (identity <90% and <94% in HA and NA, respectively).

The lineage confirmation carried out in the BV-BVRC tool for the HA indicated that it could not be classified accurately in any previous swine clade, and it was designed as an *Other-Human-2000 strain*. This assignment highlighted the human origin of the Colombian swine H3, its divergence from other swine FLUAVs, and the absence of an established phylogenetic group in which it could be allocated (Figure S1). Considering these results, A/swine/Colombia/14248/2017(H3N2) was proposed as the representative of a novel clade of the *H3-2000* lineage designated as *H3-2000.6*, following what was proposed by Anderson et al. (2021). Regarding the N2, it was noted that a relation exists with members

of the *N2-2002* lineage, being members of the *N2-2002A* clade the most similar strains. Nonetheless, their nucleotide identity regarding Colombian swine N2 was insufficient for an accurately assignment (<95%). Therefore, the NA gene of A/swine/Colombia/14248/2017(H3N2) was also designated in a novel proposed clade of the *N2-2002* lineage designated as *N2-2002C*.

In the phylogenetic tree of the HA, A/swine/Colombia/14248/2017(H3N2) was located into a major cluster that was originated from a Last Common Ancestor (LCA) dated in 1995.4 (1994.9 – 1995.9). This LCA gave rise to swine FLUAVs from Brazil (*H3-1990.5 clade*), Chile (*Other-human-1990-like*), and human seasonal viruses, that latter reached swine populations resulting in the establishment of the *H3-2000* and *H3-2010* swine lineages (Figure 3-1). The time-scaled topology suggested that Colombian swine H3N2 FLUAV was originated from an independent human-to-swine spillover that took place earlier than those that derived in the genesis of the well-described European and Asian *H3-2000* lineage. This event resulted from the introduction of a non-characterized viral predecessor that circulated in 2002.6 (2001.8 – 2003.2) and was related to a human seasonal strain from 2001. In addition to the Colombian swine H3, this predecessor also gave origin to strains that circulated in 2002.9 (2002.2 – 2003.5) and evolved in the *H3-2000* lineage in swine and as seasonal viruses in human.

Figure 3 - 1: Time-scaled phylogeny of H3 gene.[27]

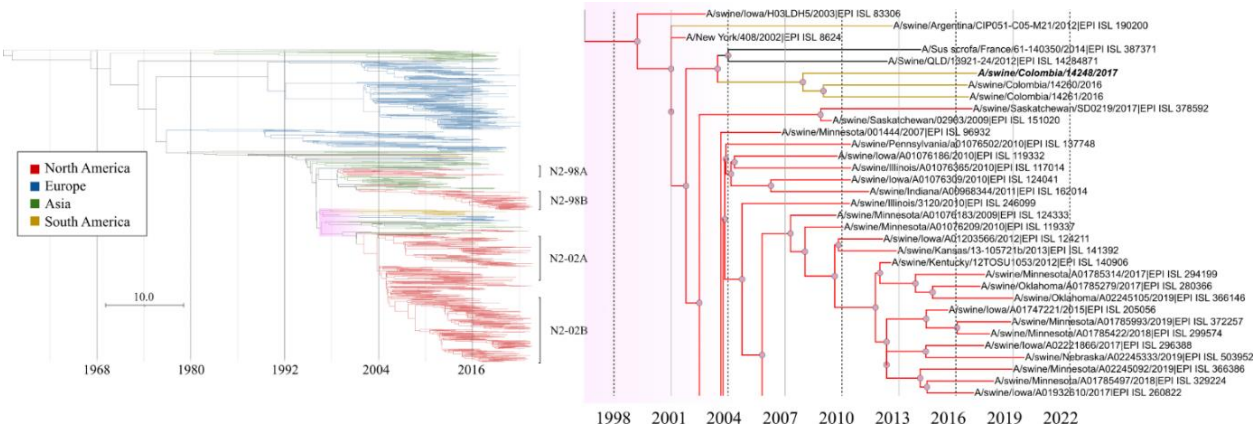


Regarding the NA gene, the results were contrasting. According to time-scaled phylogeny, the Colombian swine N2 was positioned in a predominant North American swine cluster originated from a LCA dated in 2000.3 (1999.6 – 2001.1). This LCA was related to a swine Argentinian H1N2 subtype detected in 2012 and a seasonal human H3N2 from 2002 (Figure 3-2). This LCA evolved giving rise to the *N2-2002* lineage (including *N2-2002A* and *N2-2002B*) and a divergent clade (*N2-2002C*) in which Colombian swine H3N2 and the novel H1N2 subtype (A/swine/Colombia/14260/2016(H1N2) and A/swine/Colombia/14261/2016(H1N2)) were located. In this clade, were also positioned H1N2 swine FLUAVs from France and Australia. According to the analysis, the emergence of the *N2-2002C* clade occurred simultaneously but independently from the *N2-2002A* and *N2-2002B* clades, by around 2002.3 (2001.4 – 2003.5). *N2-2002C* showed some diversification in a geographic-dependent manner, as the European and the Australian strains were closely related to each other diverging from the Colombian FLUAVs, which formed a minor subclade. It was notorious that inside this Colombian subclade, both H3N2 and novel H1N2 subtypes were slightly different, suggesting that the N2 gene from the human-origin H3N2 once in pigs evolved after the reassortment events that gave rise to the

[27] Major lineages are shown. Branches were colored according to the geographic origin of the strains. The position of the major cluster in the global tree in which the Colombian isolate was located is highlighted in a pink box (left). The Colombian H3N2 was labeled in italic and bold in the zoomed tree (right).

Colombian swine H1N2 subtype. The last ancestor of the Colombian swine H3N2 and H1N2 circulated in 2007.0 (2005.8 – 2008.2).

Figure 3 - 2: Time-scaled phylogeny of N2 gene.[28]



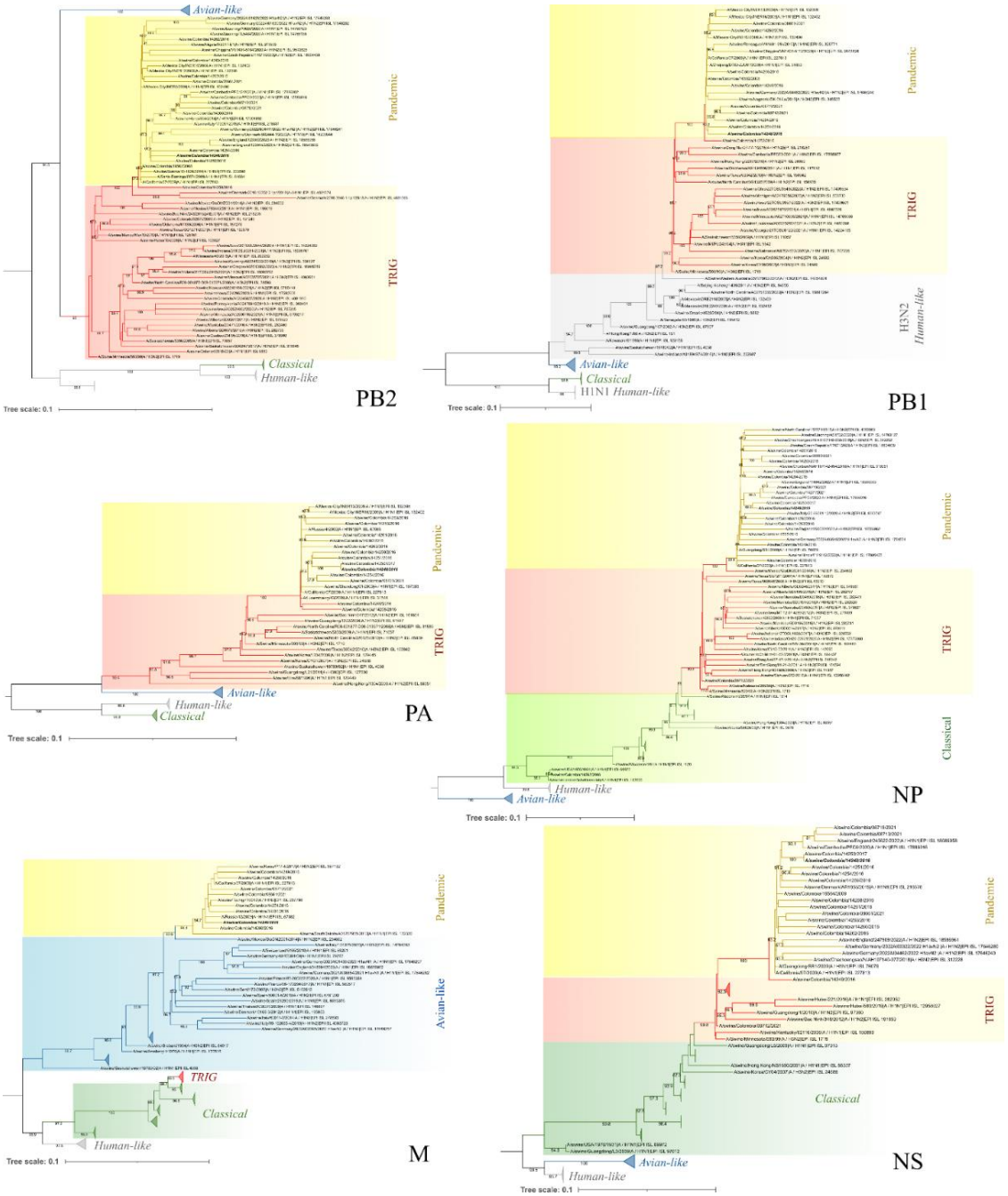
3.5.2 The internal gene constellation of Colombian H3N2 had a pandemic origin and it was mainly related to global swine FLUAVs.

The octoFLU pipeline and phylogenetic trees consistently pointed all internal genes of Colombian swine H3N2 as descendants of the H1N1 2009 pandemic cluster (pdm), being mainly related to swine FLUAVs (Figure 3-3). The PB2 gene carried by A/swine/Colombia/14248/2017(H3N2) was clustered in a fully swine group and it was

[28] Major lineages are shown. Branches were colored according to the geographic origin of the strains. The position of the major cluster in which Colombian isolate was allocated is highlighted in a pink box (left). The Colombian H3N2 was labeled italic and bold in the zoomed tree (right).

associated to FLUAVs of the H1N1pdm subtype detected in Colombia in 2016, as well as swine H1N1 and H1N2 from Europe detected since 2020. The PB1 gene was near to swine viruses from Eurasia and formed a Colombian monophyletic subclade with FLUAVs of the H1N1 subtype of both classical (1A.1) and pdm (1A.3.3.2) clades detected between 2016 and 2021. Something similar was observed for the PA gene, in which A/swine/Colombia/14248/2017(H3N2) was allocated in a Colombian subclade that contained H1N1pdm FLUAVs from the country detected in 2016, 2017, and 2021. For the NP gene, Colombian swine H3N2 resulted also in a Colombian subclade along with H1N1pdm viruses detected between 2017 and 2021, and H1N1pdm viruses from Europe and Asia. Likewise, in the NS gene of A/swine/14248/2017(H3N2) it was swine-like and it was located in a subclade along with H1N1pdm FLUAVs from Colombia, England, and Cambodia. Contrasting to the other genes, a Colombian subclade for the Matrix gene was not clearly visualized, and the evaluated strain was associated to a pdm human virus from 2009.

Figure 3 - 3: Phylogenetic trees of internal genomic segments of the Colombian H3N2.[29]



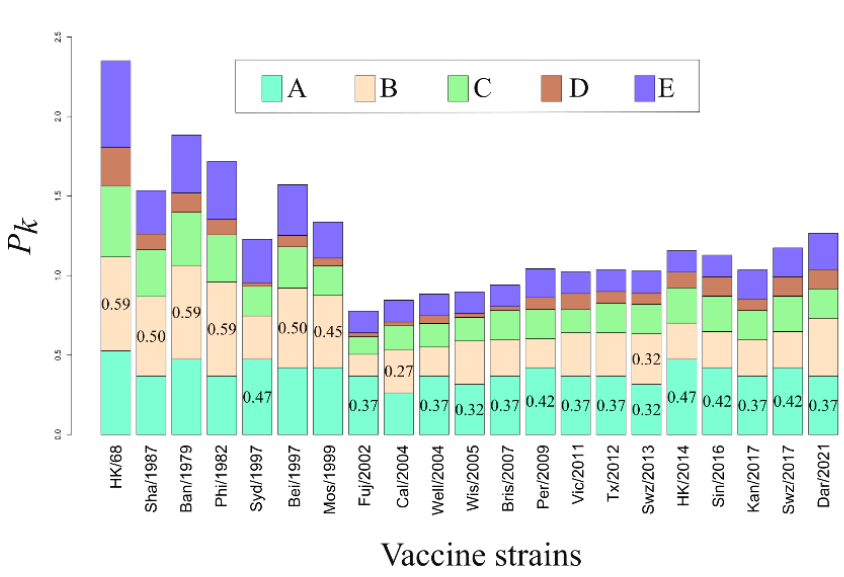
[29] The analyzed sequence is labeled in bold and italic.

3.5.3 Colombian H3N2 swine Influenza virus is antigenically divergent from seasonal vaccine strains and other swine H3 FLUAVs.

The antigenic evaluation indicated that the most divergent epitope among analyzed viruses was the B, having a mean *Pk* of 0.45 (0.0 – 0.772), followed by the A, E, C, and D which had an average *Pk* of 0.40 (0.0 – 0.68), 0.32 (0.0 – 0.59), 0.26 (0.0 – 0.63), and 0.22 (0.0 - 0.51), respectively. Regarding the Colombian swine H3N2, the greater variation was frequently calculated at the epitope A in which the mean *Pk* was 0.41 (0.26 – 0.58). In contrast, the lowest antigenic variation was mapped in the site D exhibiting a mean *Pk* of 0.13 (0.02 – 0.30). The most antigenically divergent strain from A/swine/Colombia/14248/2017(H3N2) was a member of the *H3-1970* lineage in which a *P-epitope* value of 0.77 was calculated.

When the antigenic profile of Colombian swine H3N2 was compared to vaccine strains used in humans, it was noted that a higher antigenic divergence existed as both *Pk* distances and *P-epitope* values were always above 0.22, except in the epitope D. This antigenic heterogeneity was influenced by a temporal pattern that initiated with highest distances being calculated before the 1999 season and immunodominance int the epitope B. Then, it was followed by a significant decrease in antigenic divergence during the 2000s when epitope dominance drift towards A, ending with a subsequent increase in the variation by around the 2010s (Figure 3-4).

Figure 3 - 4: Fractional differences (*Pk*) in the amino acids of each epitope between the Colombian H3N2 isolate and seasonal human H3N2 vaccine strains.[30]



The antigenic distance between the evaluated strain and swine and human seasonal vaccine FLUAVs revealed the antigenic divergence that suffer FLUAVs according to temporal and phylogenetic factors in human and swine hosts, respectively (Figure 4-5). Such divergence led to the Colombian isolate to be located near to human seasonal viruses from flu seasons of the 2000s and swine strains of the *H3-2000* lineage.

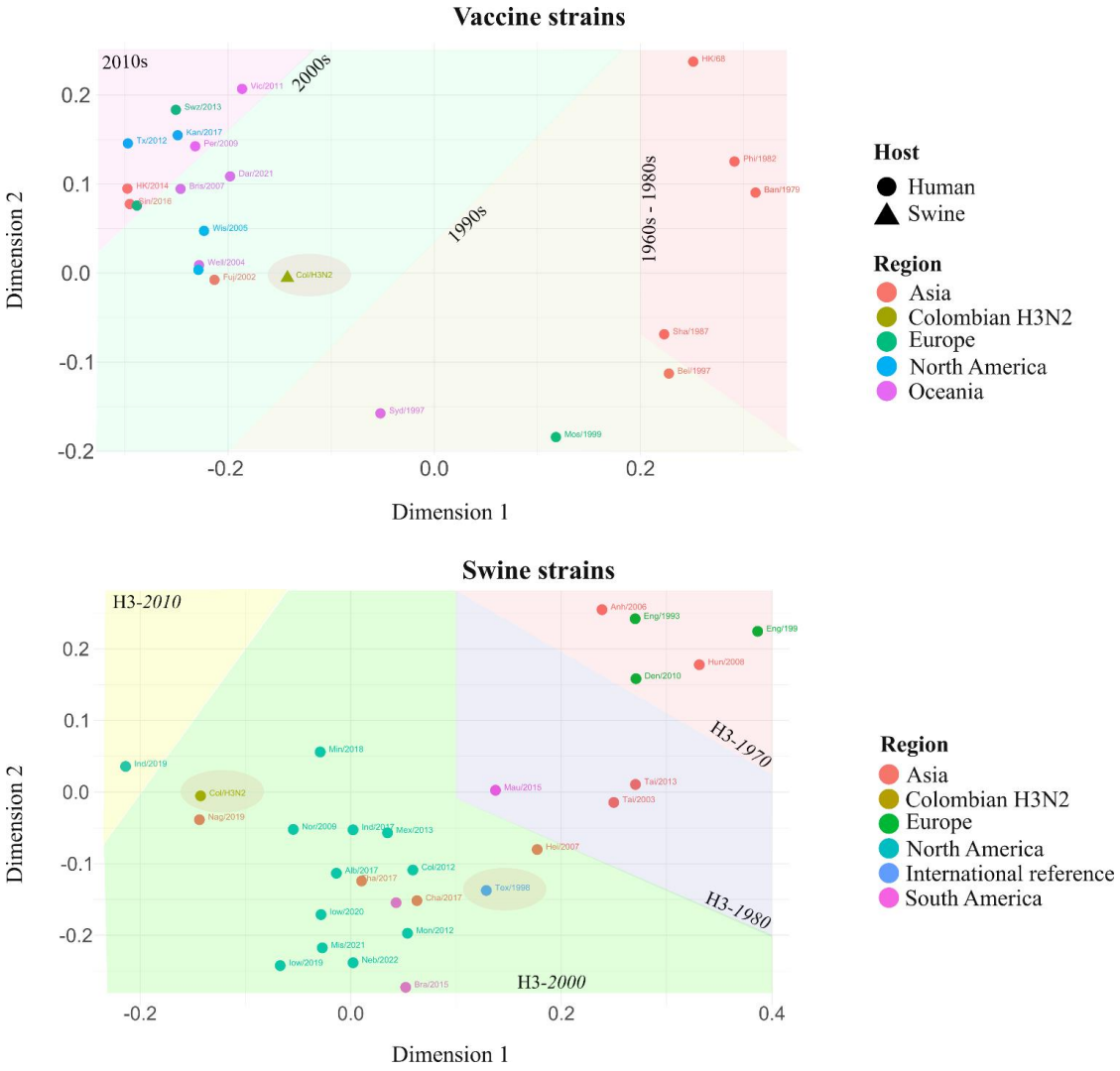
Antigenic divergence of Colombian swine H3N2 from human vaccine strains was higher (Figure 4-4 and 4-5). In this group, the most related FLUAVs were Cal/2004, Fuj/2002, and Well/2004 (A/California/7/2004(H3N2), A/Fujian/411/2002(H3N2), and A/Wellington/01/2004(H3N2), respectively), which were introduced between the 2004 and

[30] The names of human vaccine strains were simplified to include the first letters of their geographic origin and the year of their detection. *P-epitope* values are shown.

2006 flu seasons. The epitopes that dominated the variation respecting these strains were at the site A for Cal/2004 and Well/2004, and at site B for Fuj/2002. As calculated *P-epitope* values regarding all vaccine strain were always above 0.22, a lack of protection induced by vaccination against Colombian swine H3N2 was suggested. This was confirmed with the vaccine efficacy predictions made for the season 2016-2017 and 2018-2019 that indicated negative values of 70.2% and 73.8%, respectively.

Significant antigenic drift was also observed when A/swine/Colombia/14248/2017(H3N2) was compared to other swine FLUAVs (Figure 5). According to the calculated *P-epitope* values (consistently above 0.22 *vs* all swine strains), A/swine/Colombia/14248/2017(H3N2) is a novel antigenic variant. The nearest swine strain was Nag/2019 (A/swine/Nagasaki/DK-01/Tr/2019 with *P-epitope* of 0.37 at the site A) which is a member of the *H3-2000* lineage. Among swine FLUAVs, the immunodominance was in the epitope A which exhibited a mean *Pk* of 0.44 (0.32 – 0.58). B, C, and E were subordinate epitopes with *Pk* values of 0.42 (0.22 – 0.77), 0.36 (0.22 – 0.52), and 0.26 (0.22 – 0.55). Consistently with the observations made regarding human vaccine strains, the most conserved epitope corresponded to D, which showed an average *Pk* of 0.18 (0.07 – 0.30). It is worth mentioning that according to the model applied, the reference strain widely used as antigen for serological diagnostic tests in Colombia (Tex/1998) had an antigenic divergent profile that does not represent the antigenic properties of the Colombian swine H3N2 (*P-epitope* of 0.37 in the antigenic site C).

Figure 3 - 5: Multidimensional scaling (MDS) of P-epitope distances between the Colombian swine H3N2 and reference strains.[31]

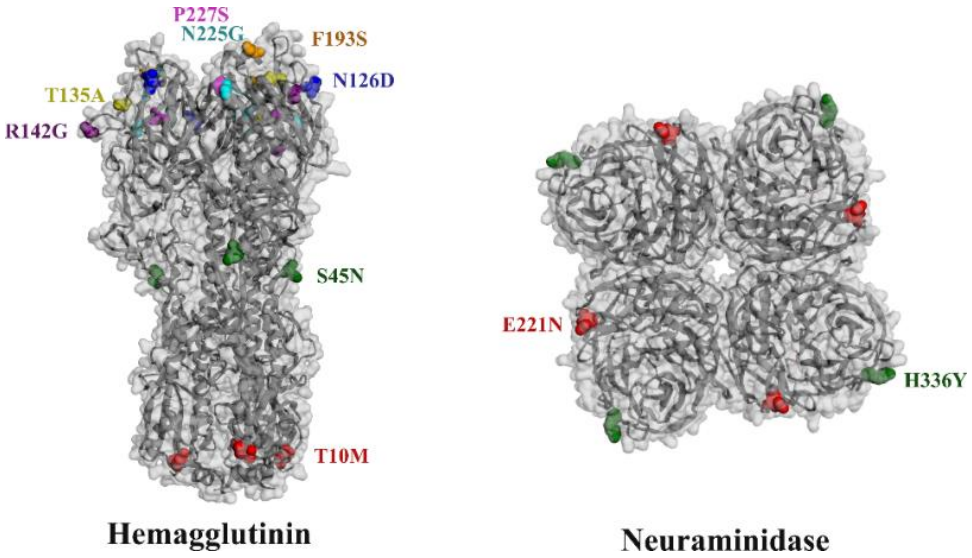


[31] In the superior map, distances between the evaluated virus and human seasonal vaccines are shown. Background colors and black labels indicate the distribution of viruses according to their temporal origin. In this map, Colombian H3N2 is highlighted in a gray circle. In the inferior map, P-epitope distances between the virus and swine FLUAVs are represented. Background colors and black labels indicate the distribution of viruses according to their phylogenetic origin. In this map, both Colombian H3N2 and the reference antigen are highlighted in gray circles. The names of strains were simplified including the first letters of their geographic origin and the numbers of the year of their detection. Conventions can be found in Table S1.

3.5.4 Colombian H3N2 carries mutations that affect posttranslational modifications and immunological recognition in the HA and NA glycoproteins.

A/swine/Colombia/14248/2017(H3N2) showed four mutations in the HA that changed the N-Glycome in the globular region of the protein (Figure 6). Three delete N-Glycosylation motifs (S10M, N126D, and T135A) and another created a new one (S45N). N126D and T134A were notorious because they were mapped in the antigenic epitope A and had been scarcely reported with frequencies of only 0.03% and 0.19% in the GISAID. Other amino acid substitution affecting antigenic sites were R142G and F193S also tracked in the epitope A and P227S in the epitope D. This last substitution as well as N225G were mapped into the Receptor Binding Domain (RBD) being part of the 200-loop and the residues that interact with the cellular receptors, respectively. Critical residues 226 and 228 showed the 226I and 228S combination, which is common in swine and is associated with the recognition of mammalian-like receptors (Rajao et al., 2019). In the NA, any molecular mark of antiviral resistance could be identified. Nevertheless, low frequent mutations related to drift in antigenic residues E221N and H336Y were observed in this glycoprotein (Figure 3-6).

Figure 3 - 6: Topographical position of mutations detected in the external glycoproteins of Colombian H3N2.[32]



3.6 Discussion

In this study we provide, for the first-time, molecular evidence of a novel swine H3N2 FLUAV in Colombia and traced its temporal origin and antigenic variation. This novel virus is the result of an unnoticed human-to-swine spillover that likely took place in Colombia at some point in 2002 (2001.4 – 2003.5). This event led to the establishment of novel H3 and N2 phylogenetic clades that were kept circulating and evolving through drift and shift in local pigs for at least 15 years, contributing to the local and global viral diversity.

[32] Proteins were modelled with Swiss-model (https://swissmodel.expasy.org) and edited with py3Dmol v2.0.4. The trimer and tetramer configuration of the Hemagglutinin and Neuraminidase are represented.

The phylogenetic characteristics of the Colombian swine H3 gene indicated that it was originated from seasonal viruses in the early 2000s, during the period when many independent human-to-swine introductions occurred leading to the establishment of the *H3-2000* lineage. Chronologically, swine H3 in Colombia corresponded to the second reverse zoonotic event that took place in that decade, appearing after the Argentinian spillover that originated the *H3-2000.1* clade but before the introductions that resulted in the development of the European *H3-2000.3* and Asian *H3-2000.2, -2000.4*, and *-2000.5* clades (Anderson et al., 2021; Cappuccio et al., 2011). Considering the observed relatedness of the evaluated swine H3N2 with members of the European and Asian *H3-2000* lineage and its phylogenetic divergence, we here proposed the existence of a novel phylogenetic American clade that was named *H3-2000.6* and it is likely restricted to Colombia. However, the possibility of its circulation in surrounding countries with limited molecular surveillance cannot be dismissed. Despite that the evidence supporting the presence of this clade is limited to the detection of A/swine/Colombia/14248/2017(H3N2), it is likely that A/swine/Colombia/14248/2017(H3N2)-like FLUAVs are currently circulating and evolving in the country. This considering that the evidence suggested its circulation for more than 10 years in Colombian pigs, where it is known that extinction of phylogroups is infrequent and as it was proved in the Argentinian *H3-2000.1* clade (Cappuccio et al., 2011; Cheng et al., 2022; Dibárbora et al., 2013). It is worthy to note, that *H3-2000.6* could be the first evidence of a second clade of the *H3-2000* lineage established in the American continent, which highlights the potential of Colombian swine viral pool as a source of non-contemporary viruses with unknown zoonotic capacities (Bhatt et al., 2013; M. I. Nelson et al., 2014; Neverov et al., 2014).

Regarding phylogenetic characteristics of the Colombian swine N2, it was notorious that significant relatedness exists with global swine viruses from France and Australia, forming a monophyletic clade that did not match any of the proposed American or European lineages (Henritzi et al., 2020; Kaplan et al., 2021). Besides, A/swine/Colombia/14248/2017(H3N2) exhibited some relatedness with an Argentinian H1N2 FLUAV that carried an NA gene derived from the introduced virus that resulted in the *H3-2000.1* clade (Pereda et al., 2011). This phylogenetic pattern is indicative that

Colombian swine N2 is derived from the same genetic pool that reached swine populations in America and other regions giving rise to *the N2-2002* lineage.

Considering that French and Australian N2 genes were allocated in the same clade that A/swine/Colombia/14248/2017(H3N2) and the Colombian H1N2 subtype (named *N2-2002C*), two theories regarding the origin of the *N2-2002C* clade are proposed. In the first scenario, independent introductions of highly related N2 genes occurred simultaneously in pigs from Colombia, Europe, and Australia, where they have been maintained evolving slowly. This is probable because the N2 gene evolves at a lower rate than the HA (Sandbulte et al., 2011). In the second scenario, a single human-to-swine introduction of the N2 gene occurred probably in Europa from where it spread into Colombian pigs. However, this is unlikely contemplating the low cross-regional movement of swine FLUAVs and regulations regarding animal importation (Cheng et al., 2022). The supportive evidence of the existence of the *N2-2002C* clade is significant considering the identification of three N2 genes from Colombia, as well as the global FLUAVs. It is probable, that *N2-2002C* clade continues circulating undetected in low-surveilled countries and underestimated in developed countries. This possibility must be evaluated in detail in posterior studies and surveillance programs.

The internal genomic constellation of A/swine/Colombia/14248/2017(H3N2) highlights the existence of unexplored reassortments between swine H1N1pdm and the introduced human-like H3N2 in Colombia. These reassortments led to the emergence of the identified H3N2 conformation constituted by human-like H3N2 with internal H1N1pdm genes, as well as the novel Colombian swine H1pdmN2 subtype. Considering the phylogenetic diversification occurred between H1pdmN2 and H3N2 at the N2 gene in 2007.0 (2005.8 – 2008.2), it is probable that during that period the first reassortments between H1N1 and H3N2 subtypes had occurred. If this is true, it suggests the presence of undetected H1N2 subtypes that carried an H1 gene of the classical lineage at some point in Colombia, as H1N1pdm emerged latter in 2009. However, this is difficult to prove considering the

absence of molecular data regarding swine FLUAV before 2008 and the lower molecular surveillance of the virus in the country. The genomic constitution of A/swine/Colombia/14248/2017(H3N2) is consistent with many observations indicating that once in pigs, human derived H3N2 subtype frequently acquire internal genes associated with swine FLUAVs because of the need for host-adapted internal genomic segments (M. I. Nelson et al., 2014; M. I. Nelson, Vincent, et al., 2012; Rajão et al., 2017).

In this study, the existence of Colombian clades in PB2, PB1, PA, NP, and NS, and their association with global FLUAV, points out the complex genetic network that exists among the viral pool in the country, which remains mostly uncharacterized. The relatedness with recent strains from Europe and Asia in PB2, NP, and NS is suggestive of cross-regional movement of viruses between the old and the new world. As this association involved only swine viruses, it is probable that internal genes arrived at Colombia through international trading of animals, nonetheless, the possibility of their introduction carried in human seasonal viruses must be considered as have been discussed before (Ospina-Jimenez et al., 2023). Regarding the characteristics of the M gene, it is likely that it was originated during the global introduction of the H1N1pdm in swine populations in 2009, as it was related to an early human H1N1pdm FLUAV (M. I. Nelson, Gramer, et al., 2012).

In relation to the antigenic characteristics of the swine H3N2, it is noteworthy that it can be classified as a new antigenic variant, as the observed *P-epitope* values regarding both human and swine FLUAVs were consistently above 0.22. Those values indicates that substantial level of antigenic drift have occurred in the HA, implying a notable antigenic mismatch with other FLUAVs (Gupta et al., 2006). This becomes more significant considering that the HA of A/swine/14248/2017(H3N2) exhibited amino acid substitutions that alter the N-Glycosylation pattern (S10M, S45N, N126D, and T135A) and that these modifications may not be adequately measured by the *P-epitope* model. Therefore, it is probable that the antigenic divergence in the HA calculated here had been underestimated and must be validated using the Hemagglutinin Inhibition-based antigenic cartography approach. This approach could shed light into a more accurately manner regarding the

antigenic properties and the effectivity of human vaccine strains against the Colombian swine H3N2, as according to our analysis, recent vaccines do not confer any protection against the virus making it a relevant threat for public health (Gupta et al., 2006).

Another captive result of this study was that the antigenic dominance of the HA in A/swine/14248/2017(H3N2) tended to be in the epitope A, which is contrary to observations made in human H3N2 FLUAVs that pointed the epitope B as the immunodominant (Broecker et al., 2018; Koel et al., 2013). This indicates that human FLUAVs once in pigs displayed a greater plasticity in the epitope A, probably due to different selective pressures acting over the HA evolution. These differential forces are likely ligated to the low selective processes that occurs in swine hosts in combination with the fitness constrains that imply the variations of amino acids in the epitope B (Wu et al., 2020). These pivotal forces can also explain the frequent loss of N-Glycosylation motifs observed in A/swine/Colombia/14248/2017(H3N2), since it is known that low posttranslational modifications of the HA are associated to animal hosts and its accumulation can cause deleterious effects (Das et al., 2011; Kim et al., 2018). For those reasons, we believe that immunodominance of site A and low levels of N-Glycosylation observed in the Colombian swine H3N2 could improve the viral fitness of the virus in pigs and are the result of its evolution in restricted non-vaccinated swine populations in the country.

In conclusion, A/swine/Colombia/14248/2017(H3N2) represents a novel phylogenetic and antigenic swine FLUAV variant that originated in the early 2000s and that persisted unnoticed among Colombian pigs for more than a decade. These findings underscore the urgent need for global molecular surveillance of animal FLUAVs, particularly in developing countries where viral dynamics and reservoirs have been underestimated as source of zoonotic and pandemic viruses and they are believed as the source of the most recent pandemic FLUAV (H1N1pdm) (Neumann et al., 2009). According to our data, Colombian swine H3N2 exhibits sufficient antigenic divergence to be considered a potential risk in the case of zoonotic swine-to-human transmission, owing to the absence of immunological

protection in the human population. For that reason, strengthening knowledge about FLUAVs in swine can contribute to pandemic preparedness and provide valuable insights into the evolutionary patterns of the virus in global animal populations. This knowledge is also essential for updating and refining diagnostic approaches also contributing to the accurately selection of representative strains to establish relevant interventions and allowing self-sufficiency of biological reagents in countries like Colombia.

3.7 Supplementary materials

Figure S1: Allocation of the evaluated H3 gene in the reference tree of the subspecies classification tool of the Bacterial and Viral Bioinformatics Resource Center - BV-BRC

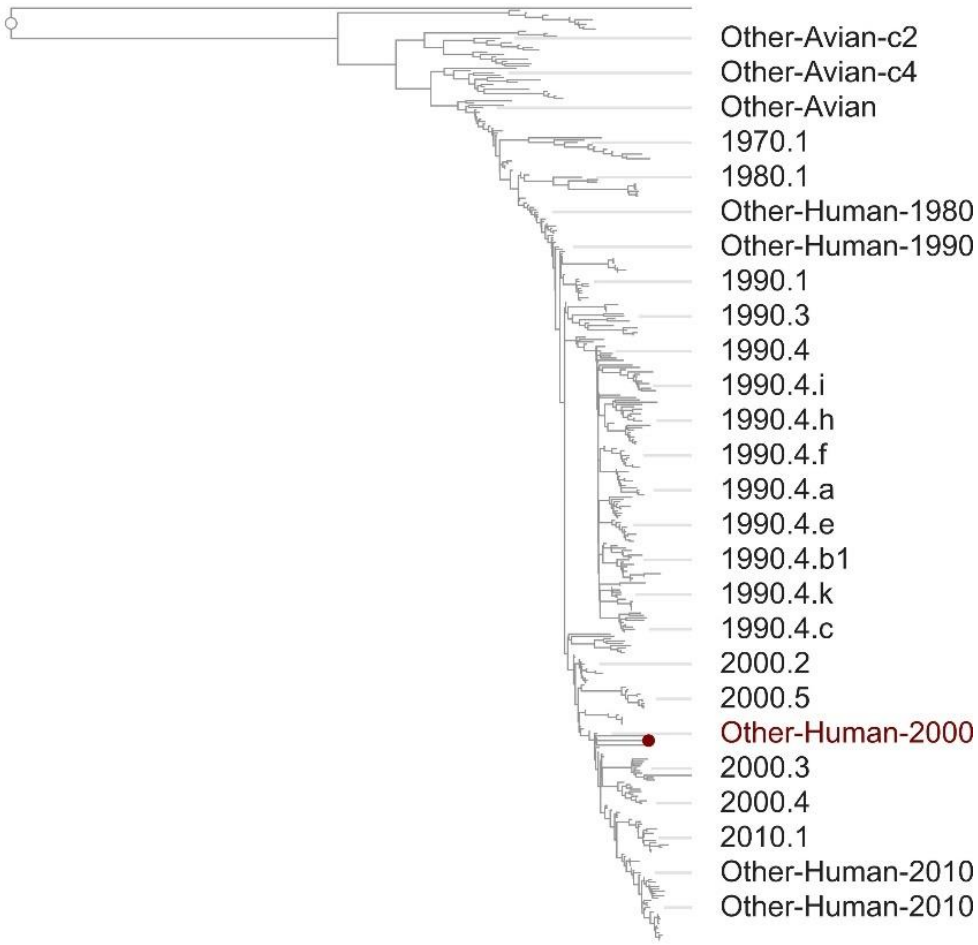


Table S1: Sequence viruses implemented for the antigenic characterization.

Isolate ID/Accession Number	Isolate Name	Subtype	Host	Abbreviature
EPI_ISL_378651	A/swine/Alberta/SD0230/2017	H3N2	Swine	Alb/2017
EPI_ISL_30177	A/swine/Anhui/01/2006	H3N8	Swine	Anh/2006
EPI_ISL_700746	A/swine/Brazil/068_15/2015	H3N2	Swine	Bra/2015
EPI_ISL_277689	A/swine/Chachoengsao/NIAH117865-070/2017	H3N2	Swine	Cha/2017
EPI_ISL_9588924	A/swine/Chile/VN1401-5054/2020	H3N2	Swine	Chi/2020
Colombian_H3N2	A/swine/Colombia/14248/2017	H3N2	Swine	Col/H3N2
EPI_ISL_130191	A/swine/Colorado/A01203748/2012	H3N2	Swine	Col/2012
EPI_ISL_179710	A/swine/Denmark/101501-1/2010	H3N2	Swine	Den/2010
EPI_ISL_129481	A/swine/England/375017/1993	H3N2	Swine	Eng/1993
EPI_ISL_129506	A/swine/England/90591/1997	H3N2	Swine	Eng/1997
EPI_ISL_77606	A/swine/Heilongjiang/10/2007	H3N2	Swine	Hei/2007
EPI_ISL_124039	A/swine/Hunan/3/2008	H3N2	Swine	Hun/2008
EPI_ISL_257035	A/swine/Indiana/A01672517/2017	H3N2	Swine	Ind/2017
EPI_ISL_366167	A/swine/Indiana/A01812290/2019	H3N2	Swine	Ind/2019
EPI_ISL_503373	A/swine/Iowa/A02478972/2019	H3N2	Swine	Iow/2019
EPI_ISL_4081702	A/swine/Iowa/A02524874/2020	H3N2	Swine	Iow/2020
EPI_ISL_328797	A/swine/Maule/VN1401-1824/2015	H3N2	Swine	Mau/2015
EPI_ISL_196015	A/swine/Mexico/9741876/2013	H3N2	Swine	Mex/2013
EPI_ISL_379928	A/swine/Minnesota/A02257619/2018	H3N2	Swine	Min/2018
EPI_ISL_14770777	A/swine/Missouri/A02636322/2021	H3N2	Swine	Mis/2021
EPI_ISL_130359	A/swine/Montana/21/2012	H3N2	Swine	Mon/2012
EPI_ISL_348522	A/swine/Nagasaki/DK-01/Tr/2019	H3N2	Swine	Nag/2019
EPI_ISL_17184137	A/swine/Nebraska/A02751434/2022	H3N2	Swine	Neb/2022
EPI_ISL_104647	A/swine/North Carolina/A01076178/2009	H3N2	Swine	Nor/2009
EPI_ISL_89183	A/swine/Tainan/103-11/2003	H3N1	Swine	Tai/2003
EPI_ISL_166449	A/swine/Taiwan/NPUST0004/2013	H3N2	Swine	Tai/2013
EPI_ISL_94059	A/swine/Texas/4199-2/1998	H3N2	Swine	Tex/1998
EPI_ISL_696536	A/swine/Thailand/CU20218/2017	H3N2	Swine	Tha/2017
AAB58297.1	A/Beijing/353/1989	H3N2	Human	Bei/1997
ABE73115.1	A/Moscow/10/1999	H3N2	Human	Mos/1999
ABG48258.1	A/Wellington/01/2004	H3N2	Human	Well/2004
ABQ10136.1	A/Sydney/5/1997	H3N2	Human	Syd/1997
ABQ97200.1	A/Hong Kong/68	H3N2	Human	HK/68
AFD64223.1	A/Fujian/411/2002	H3N2	Human	Fuj/2002
AFG99248.1	A/Shanghai/11/1987	H3N2	Human	Sha/1987
AFM68976.1	A/Bangkok/1/1979	H3N2	Human	Ban/1979
AFM72032.1	A/Philippines/2/1982	H3N2	Human	Phi/1982
AGL07159.1	A/Texas/50/2012	H3N2	Human	Tx/2012
AHX37629.1	A/Perth/16/2009	H3N2	Human	Per/2009
AIE52620.1	A/Victoria/361/2011	H3N2	Human	Vic/2011
AIU46075.1	A/California/7/2004	H3N2	Human	Cal/2004
AIU46082.1	A/Wisconsin/67/2005	H3N2	Human	Wis/2005
AIW60702.1	A/Brisbane/10/2007	H3N2	Human	Bris/2007
QIA50748.1	A/Kansas/14/2017	H3N2	Human	Kan/2017
WCF71248.1	A/Switzerland/9715293/2013	H3N2	Human	Swz/2013
WCF71352.1	A/Singapore/INFIMH-16-0019/2016	H3N2	Human	Sin/2016
WCF71375.1	A/Hong_Kong/4801/2014	H3N2	Human	HK/2014
WCF71421.1	A/Switzerland/8060/2017	H3N2	Human	Swz/2017
WEY08928.1	A/Darwin/9/2021	H3N2	Human	Dar/2021

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4. Sequence-Based Approaches Selecting Representative Strains of Swine Influenza A Virus Improves Serological Diagnosis.[33]

4.1 Introduction

Influenza A Virus (FLUAV) in pigs causes a contagious respiratory disease that leads to substantial economic losses and presents sanitary challenges for human and animal health. These effects arise from the adverse impact of infection on animal growth, its involvement in the Porcine Respiratory Disease Complex, and its zoonotic nature (Calderón Díaz et al., 2020; Parys et al., 2021; Rech et al., 2018). FLUAV has a genome composed of single-stranded segmented RNA that is prone to mutations and reassortment events that allow its permanent evolution and adaptation (Cheung et al., 2015; Marshall et al., 2013). The continuous occurrence of both processes has resulted in the evolution of many phylogenetic lineages among the swine hosts, in which three enzootic subtypes are recognized: H1N1 (lineages: classical, avian-like, and human-like), H3N2 (Lineages: 1970, 1990, 2000, and 2010), and reassorted H1N2.

The emergence of the first swine-related pandemic FLUAV in 2009 by a novel clade of the H1N1 subtype of the classical lineage (pdm09) underscored the significance of virus circulation among global swine populations. This pandemic highlighted the need for control

[33] Este capítulo será sometido para publicación en una revista indexada.

and surveillance strategies for animal FLUAVs and promoted the establishment of international initiatives such as the National Surveillance Plan for Swine Influenza Virus in Pigs in the USA, the European Surveillance Network for Influenza in Pigs (ESNIP) in Europe, and the Network of Expertise on Animal Influenza (OFFLU), which involves different countries, including some from South America. The main objective of these programs is to encourage epidemiological research on FLUAV in swine, to contribute to the accurate selection and production of relevant biological reagents, and to build a strong foundation for the preparedness for future pandemics due to FLUAV (Simon et al., 2014; USDA, 2010).

Even though the international initiatives shed lights on the phylogenetic evolution and antigenic diversity of swine FLUAV, their framework has been limited to North America, Asia, and Europe (Lewis et al., 2016). Therefore, the characteristics and evolutionary tendencies of FLUAVs in countries with low surveillance have remained in the shadows. Consequently, low or limited diagnostic capacities in these regions arise by the implementation of international biological standards, which do not represent the local virological context of swine FLUAV. This takes more relevance considering that it is known that FLUAV diversifies independently in geographically restricted swine populations (Cheng et al., 2022) and that the use of reagents from different countries can affect the performance of diagnostic assays (Buehler et al., 2014). Considering this, the World Organization for Animal Health (WOAH) encourages the permanent update of diagnostic assays to accurately represent the virological context of each region, especially for serological assays (WOAH, 2023).

Although the virus has been present for over 50 years in Colombia, surveillance of swine FLUAV is currently nonexistent. This has led to limited knowledge regarding molecular and antigenic characteristics of the virus in the territory. This absence of information has forced the use of international reference antigens with unknown representativity for serological assays such as the Hemagglutination Inhibition (HI) test. Nevertheless, according to recent studies, Colombian swine FLUAVs display significant antigenic diversity, as its being proposed the existence of five antigenic clusters for the H1 subtype (one for the Classical H1: G1A; and four for the H1pdm09: PDM09-17, PDM-CO-16, PDM-21, and PDM-CO-21) as well as the presence of a novel antigenic variant of the H3 subtype (Ospina-Jimenez et

al., 2023; Capítulo 3). In both studies, the swine H3 and four swine clusters of H1pdm09 were antigenically divergent from the international reference FLUAVs implemented as antigens for the HI assay (A/swine/Texas/4199-2/1998(H3N2) and A/swine/Iowa/15/1930(H1N1)). Considering this, the serological diagnosis in the country is probably affected by antigenic mismatches between reference FLUAVs and circulating viruses. Consequently, there is an urgent need to select local antigens that accurately depict the Colombian virological landscape for implementation in serologic assays. Nonetheless, considering that antigen selection based on standard antigenic cartography is not an option in the country, novel methodologies must be evaluated.

Therefore, the objective of this study was to assess the efficiency of a novel, rapid, low-cost, and low-labor perspective centered on sequence-based approaches for the selection and verification of field viruses as potential antigenic national standards. This study presents a pioneering pipeline suitable for countries in which selecting antigens based on standard procedures cannot be achieved. This pipeline promotes technological independence, contributes to the understanding of the antigenic evolution of swine FLUAV, and strengthens diagnostic and biosecurity capacities in developing countries. The study also emphasizes the critical importance of ongoing surveillance and the determination of antigenic properties in locally circulating FLUAVs to increase diagnostic capacity, improve animal health, and strengthen the implementation of pandemic preparedness plans.

4.2 Materials and methods

4.2.1 Viruses and selection of candidates.

Thirty-eight Colombian swine FLUAVs (four classical H1N1, 29 H1N1pmd09, two H1pdm09N2, and one H3N2) antigenically characterized through sequence-based approaches were considered (Ospina-Jimenez et al., 2023; Capítulo 3). These FLUAVs came from nasal swabs and lung samples collected between 2008 and 2021 from commercial pig farms located in eight Colombian regions. Isolates or original samples were

available in the virological repositories of the Colombian Agricultural Institute (ICA) and the Molecular Biology and Virology Laboratory of the Universidad Nacional de Colombia. Most of the considered FLUAVs were already isolated at the beginning of this study. The H1 subtype viruses were previously characterized by the methodology developed by Anderson et al. (2018), and the H3 by the approach designed by Gupta et al. (2006) and Bonomo et al. (2019).

Based on the antigenic analyses, seven antigenic candidates were selected and evaluated. This number was chosen to represent the five H1 antigenic clusters predicted by Ospina-Jimenez et al. (2023), the H1pmd09N2 subtype, and the novel swine H3N2 (Capítulo 3).

The selection of candidates in the H1 subtype consisted in a two steps approach. First, pan-epitope consensuses encompassing only amino acids in the epitopes of the HA (Sa, Sb, Ca1, Ca2, and Cb) were constructed for each antigenic cluster. Then, members of each cluster were compared to the consensus of the corresponding antigenic group. Strains with the lowest divergence respecting consensuses were selected as representatives. In cases where multiple FLUAVs displayed equivalent low divergence, priority was given to strains with the most recent detection dates. For this analysis, consensuses were generated with the Cons Tool from the EMBOSS v6.6.0.0 (https://www.ebi.ac.uk/Tools/msa/emboss_cons/; accessed on 8 March 2023) and the comparisons were achieved by the pairwise distance analysis using the *seqinr* v4.3 package in the RStudio software v4.3.2.

For the H1pdm09N2 subtype, the selection was carried out considering the strain with the highest amino acid divergence from the consensus in which the subtype was assigned (PDM-09-17). In the case of the H3N2, as there was only one virus available, it was included as candidate for this subtype.

4.2.2 Viral isolation and production.

Isolation attempts for non-isolated candidates were performed from the original samples by allantoid cavity inoculation of 10-day-old SPF embryonated chicken eggs. Once candidates were isolated, their replicative capacity was tested into two substrates for the standardization of viral seed production. The evaluation was carried out by comparing viral propagation in 10-day-old SPF embryonated chicken eggs (ECE) and Madin-Darby Canine Kidney (MDCK) cell monolayers following standard procedures (Eisfeld et al., 2014; WOAH, 2023). The replicative capacity of candidates was assessed comparing viral titers harvested from ECE and MDCK using the Hemagglutination test (HA) with 1% chicken red blood cells. Samples that resulted positive for the HA test were confirmed as positive for FLUAV by an RT-PCR targeting the Matrix gene. Viral seeds were produced in the substrate where FLUAVs showed the highest HA titer. The harvested seeds were stored at -70°C until their use.

4.2.3 Antigenic stability verification of viral seeds

To ensure antigenic stability in the viral seeds, the acquisition of laboratory-induced mutations was determined through sequencing of the HA gene. For this purpose, viral RNA was extracted using the RNeasy Mini Kit (Qiagen®). The RT reactions were performed with the M-MLV Reverse Transcriptase kit (Invitrogen™) and the uni-12 primer (5'-AGCAAAAGCAGG-3'). cDNA was then used for amplification of the HA gene. PCRs for viruses of the H1 subtype were conducted in two separated reactions that allowed the amplification of the gene in two overlapping fragments. For this reactions, universal primers reported by Hoffmann et al. (2001) and internal primers kindly donated by the Influenza Research Laboratory of the University of Maryland—USA were employed (Table S1). In the H3 subtype, the amplification was achieved in a single reaction that contained only the external primers reported by Hoffman et al. Amplifications were carried out in 12.5 µl master mix reactions that contained 1 µl of cDNA, 1X of 10X PCR Buffer (-MgCl2), 3 mM of MgCl2, 0.4 mM of dNTPs, 0.6 µM of each primer, and 0.05 U/µl the Platinum™ Taq DNA Polymerase (Invitrogen™), that were run at 94°C for 4 min, 40 cycles of 94°C for 40 s, 56°C for 30 s, 72°C for 3 min, and 72°C for 10 min. PCR products were sequenced by the Sanger method at the Instituto de Genética of the Universidad Nacional de Colombia. Internal

primers that divide the gene into two overlapping regions were included in the sequencing reaction (Table S1) to cover the length of the H3 product (~1700 pb). Sequences were assembled using the Geneious software v2023.2.1 (Dotmatics).

Two approaches were used to confirm antigenic stability in the HA gene of the viral seeds. The first attempt consisted in a BLAST analysis comparing the nucleotide identity of sequences in the seeds and the original sequences used for the antigenic characterization. This analysis was carried out in the BLAST tool of the National Center for Biotechnology Information - NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 27 December 2023). For the second approach, using the Geneious software v2023.2.1 (Dotmatics), we aligned the HA proteins coded by the viral seeds with protein sequences analyzed during the antigenic characterization.

4.2.4 Production of antigens for Hemagglutination Inhibition assay

To produce antigens for serological assays, viral seeds were inoculated into the substrates that showed a better replicative performance (ECE or MDCK cells). After an incubation period of 72 hours, the antigens were harvested and titrated using the HA assay. The presence of the FLUAV was confirmed through an RT-PCR targeting the matrix gene mentioned previously. The produced antigens were aliquoted and stored at -70°C until their use.

4.2.5 Verification and comparison of antigens in the Hemagglutination Inhibition assay.

To evaluate the effectiveness of the proposed antigens in the HI assay, two sets of swine serum samples were tested. The first set (Set 1) consisted of 20 serum samples collected in 2021 and 2022 that were available in the serological repository of the ICA. This set included seropositive and seronegative samples tested with the Swine Influenza Virus

Antibody ELISA kit (IDEXX) (positivity by ELISA: 70%). Serum samples of sows and nursing-fattening pigs from farms located in Antioquia, Cundinamarca, Meta, Huila, Cauca, and Valle del Cauca were evaluated. The second set of samples, referred as Set 2, consisted of 54 field samples collected in 2023 from commercial farms with historic episodes of respiratory disease during surveillance activities carried out by the ICA. This second set was also designed to include farms with both, seropositive and seronegative animals by the Swine Influenza Virus Antibody ELISA kit (IDEXX) (positivity by ELISA: 63%). For optimal performance, fresh dilutions of antigens with eight Hemagglutination Units (HAU) in 50 µl (4 HAU/25 µl) were confronted with pre-treated serum samples according to established protocols (WOAH, 2023).

To assess the repeatability, three pools of sera (positive, intermediate, and negative pools) using samples from the first set tested were prepared. Samples with HI titers ≥ 1:80 and ≥ 1:40 against the H1pdm09 and the reference antigens, respectively were used for the preparation of the positive pool (CP). An intermediate pool (CPD) was prepared using samples with titters ranging from 1:40 – 1:80 for H1pdm09 and 1:10 – 1:40 for the international reference antigen. The Negative pool (CN) included samples that were negative against all, the proposed and the reference antigens. Following the WOAH recommendations (2024), each pool was tested 20 times with both the proposed and the reference antigens, on different days and by two analysts.

4.2.6 Statistical analysis.

Differences in the HI assay performance was compared using the Friedman and Pairwise Wilcoxon tests for the H1 subtype, while for the H3 subtype a paired Wilcoxon test was conducted. The global concordance in the assignation of samples as positive or negative was evaluated using the Fleiss' Kappa and Cohen's Kappa tests for the H1 subtype. For these analyses, HI results were categorized into positive or negative considering as positive samples with HI titters ≥1:40.

The repeatability was estimated using coefficients of variation (CV). Global and intra-assay repeatability were calculated for the 20 repetitions and the intra-assay repetitions, respectively. All analyses were carried out using the RStudio software v4.3.2.

4.3 Results

4.3.1 Selected candidates had a low pairwise distance from consensuses and replicated well only in SPF embryonated chicken eggs.

Selected candidates for the H1N1pdm subtype for the PDM09-17, PDM-CO-16, PDM-21, and PDM-CO-21 clusters were A/swine/Colombia/14255/2017(H1N1) (Ag14255), A/swine/Colombia/14268/2016(H1N1) (Ag14268), A/swine/Colombia/08721/2021(H1N1) (Ag08721), and A/swine/Colombia/14269/2021 (H1N) (Ag14269). These viruses showed a pairwise distance of 0.0 from consensuses within their respective cluster.

The H1N2 subtype strain selected was A/swine/Colombia/14261/2016(H1N2) (Ag14261). It belongs to the PDM09-17 cluster and it has a pairwise distance of 0.32 (four amino acids) from the consensus representing that cluster. For the antigenic group corresponding to the classical H1N1 (G1A), the first selected strain was A/swine/Colombia/08712/2021(H1N1), nevertheless, it could not be isolated. Therefore, a previous FLUAV of the same antigenic cluster (A/swine/Colombia/0401/2008(H1N1) (Ag0401) was selected. Although this strain was older, according to the sequence-based approaches there are no antigenic variations in any member of the cluster and any strain could be representative (Ospina-Jimenez et al., 2023).

All candidates showed a higher replication rate in ECE as compared to MDCK cells. When FLUAVs were inoculated in ECE, it was evident that harvested allantoid fluid contained virus with higher HA titers, typically greater than 1:64. In contrast, when they were

inoculated in MDCK cells, the maximum HA titers obtained were 1:2. Therefore, the viral seeds and antigen production were carried out using ECE.

4.3.2 Viral seeds were antigenically stable and showed only minor mutations.

High-quality sequences of the HA gene were accomplished from all viral seeds (mean coverage of 86.8%). In these viruses, single mutations were observed except for Ag0401 and Ag14269 which had an identity of 100% with the original sequences. Sequences from Ag14255, Ag12461, Ag14268, Ag08721, and Ag14248 had 99.93%, 99.94%, 99.94%, 99.93%, and 99.93%, nucleotide identity with the original sequences respectively, and each one displayed a single mutation. The viral seeds Ag14268 and Ag14248 acquired synonymous mutations that did not affect the amino acid sequence of the HA (G652A and C1082A). Non-synonymous mutations were detected in the viral seeds Ag14255, Ag12461, and Ag08721. The mutations in Ag12461 and Ag08721 were mapped in the amino acid 190 (V190L and S190R, respectively), which is located outside the epitopes and did not change the antigenic profile of the HA. A mutation in Ag14255 was tracked near the Receptor Binding Domain (Q223R) but it was also found outside the antigenic regions. It was found that the viral seeds preserved the antigenic profile of the original sequenced strains.

4.3.3 Proposed antigens resulted in the detection of higher antibody titers than the reference strains.

The results obtained from assays conducted with both serum sets indicated that the proposed antigens tend to exhibit higher antibody titer values, which was statistically significant (H1: Friedman test $p < 0.01$; H3: Wilcox test $p < 0.01$), compared to the reference strains for both H1 and H3 subtypes (Table 4-1 and Figure 4-1). For the H1 subtype, the viral candidates showed titers equal to or above 1:40 more frequently than A/swine/Iowa/15/1930(H1N1), detecting more positive samples (Figure 4-1 and Table 4-1). Most of samples for the H3 subtype were negative. However, when the proposed antigen

Ag14248 was used, it resulted in the detection of a positive sample and samples with low antibody titers (1:10) (Table 4-1).

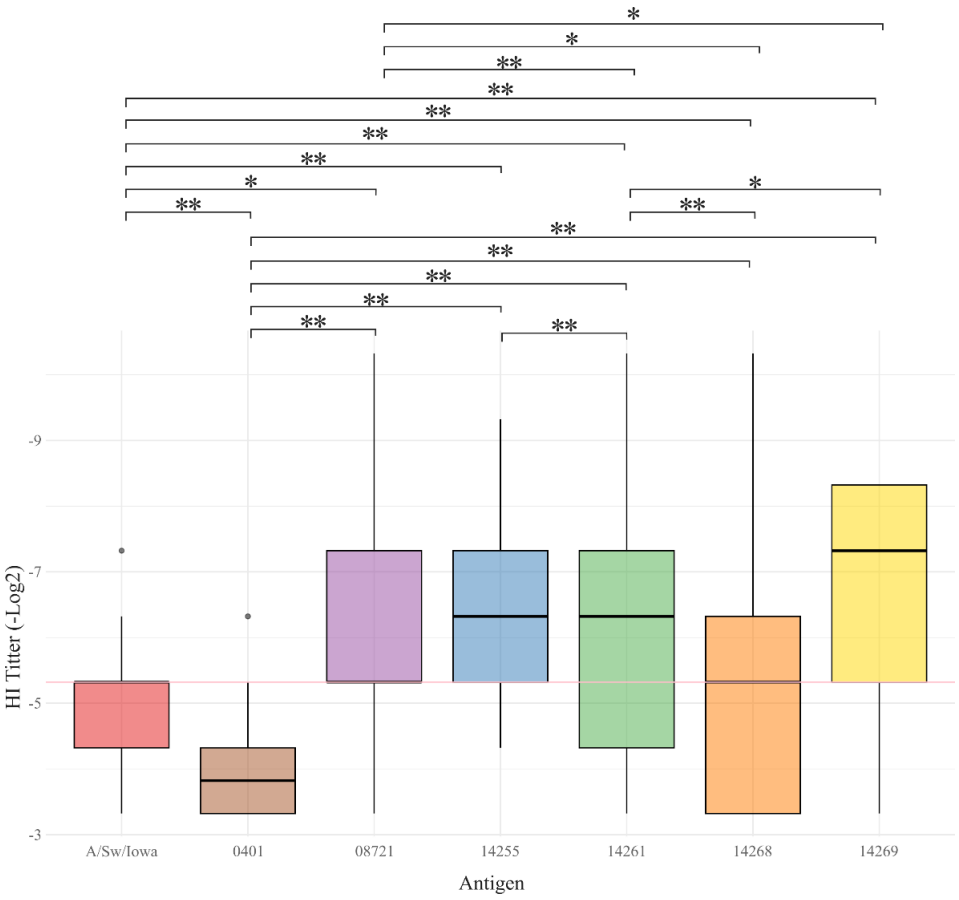
Although the proposed viral candidate of the classical clade (Ag0401) and the reference A/swine/Iowa/15/1930(H1N1) had the same amino acid sequence in the epitopes of the HA, they exhibit significant differences in the HI assay performance (Pairwise Wilcox test $p < 0.005$), and the samples tested with the Ag0401 antigen showed a low-level antibody titer (Table 4-1 and Figure 4-1).

Table 4 - 1: Proportion of positive samples according to HI assays using the proposed and the reference antigens.

Subtype	Antigen	Set 1	Set 2	Total
H1N1	Reference: A/swine/Iowa/15/1930(H1N1)	35%	1.9%	10.8%
H1N1pdm09	Ag14255	70%	3.7%	21.6%
	Ag14268	60%	5.6%	20.3%
	Ag14269	70%	3.7%	21.6%
	Ag08721	60%	3.7%	18.9%
Classical H1N1	Ag0401	5%	1.9%	2.7%
H1pdm09N2	Ag14261	70%	9.6%	25.7%
H3N2	Reference: A/swine/Texas/4199-2/1998(H3N2)	0%	0%	0%
	Ag14248	0%	1.9%	1.4%

Figure 4 - 1: Results of HI assays comparing the proposed national antigens and the international reference antigen A/Sw/Iowa (A/swine/Iowa/15/1930(H1N1)).[34]

[34] The threshold for considering a sample as positive is highlighted in a red line. *$p < 0.05$; **$p < 0.01$.



Two groups of the proposed antigens of the H1N1pdm09 subtype were identified, with significant differences between them. The first group consisted of Ag08721, which was statistically similar to Ag14255 (Pairwise Wilcox test $p > 0.05$). The second group included Ag14269, which was comparable to Ag14255 and Ag14268 (Pairwise Wilcox test $p > 0.05$).

The H1pdm09N2 subtype antigen demonstrated different results from all other H1pdm09 candidates, indicating that Ag14261 is a unique antigen (Pairwise Wilcox test $p < 0.05$).

4.3.4 There was some concordance between candidates and the reference strain results for the H1 subtype.

The assays showed moderate agreement in the detection of seropositive samples in the H1 subtype ($k = 0.673$; $p < 0.005$). Paired concordance allowed the grouping of three antigens (Ag14255, Ag14269, and Ag08721) that displayed the highest agreement in the categorization of samples ($k > 0.9$; $p < 0.005$). Additionally, there was some level of agreement between the candidate antigens Ag14261 and Ag16268 with all the other pandemic candidates ($k > 0.8$; $p < 0.005$).

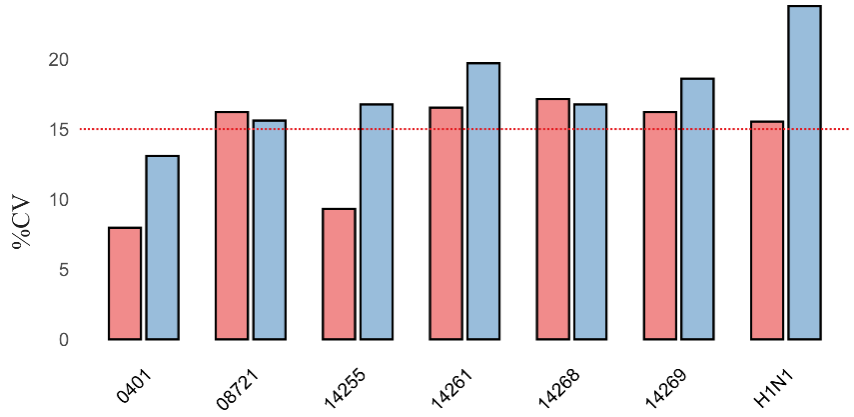
As expected, the lowest concordance was determined between candidates of the H1pdm09 subtype and Ag0401, among which a $k < 0.2$ was consistently calculated ($p < 0.05$). This finding contrasted to the agreement for H1pdm09 antigens and the reference strain that displayed a moderate categorization harmony ($0.5 < k < 0.7$; $p < 0.005$). The congruence between Ag0401 and A/swine/Iowa/15/1930 was weak with a $k = 0.373$ ($p < 0.005$).

The categorization in the H3 subtype could not be assessed considering that most of the samples were negative for both Ag14248 and the reference H3N2 antigen.

4.3.5 Antigens of the H1 subtype exhibited similar variation to the reference strain.

The results of virus candidates and the reference strain of the H1 subtype were characterized by significant fluctuations with CVs frequently above 15% when assessing both CP and CPD pools (Figure 4-2). Notably, CVs in repetitions of the CN were always 0%, as this pool consistently resulted in negative lectures. The Ag0401 candidate demonstrated the most repeatable results, with an overall CV of 8% and 13% for the CP and CPD pools, respectively.

Figure 4 - 2: Global coefficients of variation of the results in positive (red) and intermediate (blue) serum samples confronted to proposed and reference (H1N1) antigens.[35]



The intra-assay variability for CP pool tended to be low, with the greatest mean CV (4.6%) identified in the candidate Ag14261 (0% - 7.9%). Higher intra-assay repeatability values were observed in antigens Ag14268, Ag14269, Ag08721, and Ag0401 (CV = 0%). Some variation was present in Ag14255 and the reference strain which exhibited mean CVs of 1.7% (0% - 6.9%) and 1.5% (0% - 6%), respectively.

The higher intra-assay repeatability was measured in the CPD pool. Using this sample, the greatest variability was 6.1% and was restricted to the antigen Ag0401. Antigens Ag14255, Ag14261, Ag14268, Ag14269, and A/swine/Iowa/15/1930 showed the most reproducible values accounting for CVs of 0%.

[35] Results are presented as percentage. The threshold of 15% encouraged for intra-assay repetitions of serological assays recommended by the World Organization for Animal Health is highlighted as a discontinuous red line.

In the CN pool, all measurements showed 100% repeatability (CV = 0%). No repeatability assessment could be conducted for the Ag14248 and A/swine/Texas/4199-2/1998(H3N2) viruses of the H3 subtype due to the lack of positive samples.

4.4 Discussion

In this study, we present evidence for the application of sequence-based methodologies for selecting representative field FLUAVs as potential national reference antigens for diagnosis. These approaches proved to be beneficial for enhancing the performance of serological assays and demonstrated the potential to produce locally relevant biological reagents. Countries facing laboratory limitations can improve the selection of local antigens by employing these methodologies, thereby enhancing the diagnostic performance to meet the mandatory recommendations outlined by the WOAH (2024). Additionally, it helps to reduce the number of strains necessary for the evaluation through standard approaches, thus reducing technical and financial costs for the surveillance of antigenic characteristics of swine FLUAV.

In this study, it became evident that differences exist in antibody titers detected using the characterized local viruses and the international reference antigens. This highlights weaknesses in the serological diagnostic capacity of swine FLUAV in Colombia. The currently implemented antigens tended to yield negative results for samples that were positive for the proposed candidates. These false-negative results from antigenic mismatches between field and reference viruses arise from the independent variation of FLUAV in Colombian swine populations (Cheng et al., 2022; Lewis et al., 2016).

The sequence-based approach implemented in this study showed good performance in terms of serological results. This is supported by the fact that, as predicted, the proposed antigen for the H3N2 subtype and most of the candidates of the H1 subtype were significantly different from the reference strains currently used for HI assays in Colombia (Ospina-Jimenez et al., 2023; Capítulo 3).

Differences observed in the swine H1 antigenic clusters within the country were in line with the predictions made by Ospina-Jimenez et al., (2023). When comparing antigens from clusters with mean Antigenic Distances (AD) greater than 4.0 Antigenic Units (AU), significant differences were found in performance of the HI assay. This supports the hypothesis that a threshold of 4.0 AU for considering swine FLUAVs of the H1 subtype as antigenically divergent is largely accurate. This was demonstrated by the comparison of classical antigens (Ag0401 and A/swine/Iowa/15/1930(H1N1)) with all pandemic candidates, as well as candidates of the PDM-21 and PDM-CO-16 clusters (Ag08721 and Ag14268, respectively; AD > 4.0 AU) (Ospina-Jimenez et al., 2023). However, it is necessary to validate this value by using the gold-standard HI-based antigenic cartography. Non-significant differences existed with the antigens of the PDM-CO-21 and PDM-CO-16 clusters (Ag14269 and Ag14268), despite having a mean AD of 5.1 AU. This unexpected result could be a consequence of the evaluation of serum samples with antibodies against more than one antigenic cluster. This possibility takes relevance because PDM-CO-21 and PDM-CO-16 clusters were detected in animals from the same region (Antioquia) where cocirculation of both clusters is plausible.

As expected, higher seropositivity rates were found for antigens of the H1N1pdm09 clade, while reactivity to classical H1N1 remained low. This is in line with molecular observations that suggest the dominance of H1N1pdm09 FLUAVs and the lower circulation of classical H1N1 in Colombia since 2009 (Ciuoderis et al., 2019; Flórez Ramos et al., 2018; Osorio-Zambrano et al., 2022; Ospina-Jimenez et al., 2023; Ramirez-Nieto et al., 2012). The low seroreactivity to the H3N2 subtype was associated with the low molecular evidence of its presence in Colombian pig farms.

Based on the HI performance for the H1N1pdm09 antigen, we proposed a refinement in the selection of potential candidates, and ultimately chose Ag14269 for this clade in Colombia. This selection is based on the absence of statistical differences in antibody titers detected when Ag14269 was compared with antigens of the PDM09-17 and PDM-CO-16 clusters, and its significant concordance in the categorization of samples as positive or

negative (k = 0.9) with candidates of the PDM09-17 and PDM-21 antigenic groups. Additionally, Ag14269 was the antigen that detected the highest antibody titers in the evaluated samples (Figure 4-1). Therefore, it could be selected as the national antigenic reference virus to stand for the H1N1pdm09 subtype in rutinary serological diagnosis in Colombia.

The disparity detected when comparing the classical candidate Ag0401 and A/swine/Iowa/15/1930(H1N1) was notable considering that the calculated AD between both was 0.0 AU. In the assays, A/swine/Iowa/15/1930(H1N1) resulted in highest seropositivity rates than Ag0401. This unexpected outcome can be elucidated by two primary factors. In the first place, it is plausible that A/swine/Iowa/15/1930(H1N1) displayed low-grade cross-reactivity with FLUAVs of H1N1pdm09, and most titers corresponded to heterologous antibodies. This is probable considering that H1N1pdm09 is the dominant FLUAV in Colombia and that this clade has a proved phylogenetic and antigenic relatedness with ancestral classical H1N1 FLUAVs (Garten et al., 2009; Min et al., 2010). On the other side, it must be considered that Colombian classical H1N1 likely diverged from ancestral strains and from the H1N1pdm09 clade. This hypothesis is proposed considering the sustained circulation of classical H1N1 in the country for over three decades without vaccination, which could lead to the acquisition of amino acid substitutions outside the canonical epitopes of the HA. These changes could alter the shape, biophysical characteristics, or posttranslational composition make up of the glycoprotein making it antigenically divergent (Hu et al., 2020; Stray & Pittman, 2012). All these factors and the lower molecular detection of classical H1N1 in the country could explain the low reactivity for Ag0401 compared to A/swine/Iowa/15/1930(H1N1) in the serum sets assessed. It should be noted that we included the only four classical H1N1 viruses available, which may not be enough to fully represent the local diversity of this subtype in the country. As a result, further research is needed to better understand this virus and robust the selection of representative antigens for this subtype. However, based on the findings of this study, Ag0401 could be proposed as a representative of the classical H1N1 virus subtype for serological assays in Colombia.

In the case of the H1pdm09N2 subtype, significant differences were noted between the proposed candidate and the other tested strains. In this subtype, the highest seropositivity rate among samples was detected. This seropositivity is likely due to a significant circulation of H1N2 FLUAVs in local swine populations as has been noted in other regions, where it is the second most common subtype detected (Henritzi et al., 2020; Simon et al., 2014). However, this has not been proven in Colombia and must be evaluated in detail. Although Ag14261 carried an HA assigned to the same antigenic cluster as Ag14255 (PDM09-17), they displayed different results in the HI assays, indicating sufficient antigenic divergence between the two antigens. This divergence appears to be insufficiently explained by amino acid variations in the epitopes (only four amino acids). As a result, it is postulated that the entire HA gene of Ag14261 might have undergone different evolutionary pathways compared to other members of the same antigenic cluster. This is proposed considering that it has been shown that the different subtypes of NA provide epistatic factors that alters the HA (Hu et al., 2020; Liu et al., 2022). Alternatively, it is also feasible that the four substitutions observed in glycoprotein caused substantial alterations in its biochemical properties or affected crucial residues that were not accurately weighted by the linear nature of the sequence-based methods (Quan et al., 2019; Wang et al., 2021). In any case, Ag14261 should not only be considered a mandatory antigen for inclusion in routine HI assays to represent the H1pdm09N2 subtype in Colombia but should also be evaluated through gold-standard antigenic cartography.

The representative results for the H3N2 cannot be measured considering that most of samples were seronegative against this subtype and the proposed antigen corresponded to the only available swine H3N2 FLUAV in the country. Nevertheless, significant differences in antibody detection could be calculated between Ag14248 and the reference A/swine/Texas/4199-2/1998(H3N2). With this limited data, two main conclusions can be drawn. In the first place, it became evident that the international reference antigen lacks relevance for serological diagnosis in the local context, as all tested samples consistently yielded negative results. Furthermore, the positive serum control used for routinary HI assays only reacted with A/swine/Texas/4199-2/1998(H3N2) but not with Ag14248. In the other place, there was evidence of circulation of Ag14248-like FLUAVs at a marginal level in Colombian pigs, which may explain the detection of one positive sample and the

presence of samples with low antibody titers (titers = 1:10). Based on these data, Ag14248 is postulated as a national antigen for the H3N2 subtype, as it allowed the detection of antibody titers that the reference strain did not. However, further research must be conducted on in this subtype to support this postulation.

The repeatability of antibody titers detected by the proposed candidates and the reference strain in the H1 was comparable. This indicates that the HI assays had similar performance for this subtype, regardless of the antigens used. The calculated CVs for the 20 repetitions performed in CP and CPD were as expected considering the intrinsic variability of the HI assay. Therefore, it is necessary to standardize and qualify the assay for each antigen implemented (Noah et al., 2009; Sawant et al., 2023; Zacour et al., 2016). These processes allowed to overcome the inter-assay variability due to uncontrollable factors such as sample matrix composition, red blood cell type and lot-to-lot variation, NA-mediated hemagglutination, and environmental conditions (Hooper et al., 2015; Long et al., 2004; Sawant et al., 2023). Nevertheless, as all the candidates displayed inter-assay CVs below 20%, they were considered antigens with a precise performance (Sawant et al., 2023). Furthermore, the proposed antigens displayed repeatable results as none of the samples (CP, CPD, or CN) exceeded the intra-assay threshold of 15% for the CVs (WOAH, 2024).

In summary, the proposed pipeline through sequence-based approaches evaluated in this study facilitated the identification of six potential antigenic candidates of the H1 subtype and one of the H3 subtype. The verification with the HI assay confirmed significant variations between the proposed candidates and international reference antigens. Consequently, three final candidates for the H1 subtype (Ag14269 for H1N1pdm, Ag14261 for H1pdm09N2, and Ag0401 for classical H1N1) and one for the H3N2 (Ag14248) subtype appeared as potential national representative antigens. Although these candidates require validation through gold-standard methodologies, they demonstrated to improve the performance of the HI assay, giving enough value to consider them for inclusion in current serological protocols in the country.

In conclusion, the proposed methodology has the potential to improve diagnostic and control strategies for swine FLUAV by better detection of infected animals. This will allow the implementation of more effective biosecurity measures. Moreover, this methodology contributes to the understanding of the antigenic diversity of swine flu viruses in Colombia and worldwide, providing valuable insights for pandemic and zoonotic influenza preparedness plans.

4.5 Supplementary Material

Table S1: Primers used for sequencing of the HA gene of the proposed viral candidates.

Subtype	Primer	Sequence (5'-3')	Fragment	Size pb
H1	BmHA1F	TATTCGTCTCAGGGAGCAAAAGCAGGGG	HA1	931
	SwHA931R	TCTGAAATGGGAGGCTGGTGTT		
	SwHA752F	TAGAGCCGGGAGACAAAATAACAT	HA2	1026
	BmNs890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT		
H3	BmHA1F	TATTCGTCTCAGGGAGCAAAAGCAGGGG	HA1	1095
	H3 Reverse	GATGCCTGAAACCGTACCA		
	H3 Forward	CGCAATMGCAGGTTTCATAGA	HA2	649
	BmNs890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT		

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5. Conclusiones y recomendaciones

5.1 Conclusiones

Este estudio presenta, por primera vez, evidencia de la diversidad antigénica del virus de Influenza A en porcinos del país, destacando la continua necesidad de comprender no solo las características moleculares de este virus, sino también sus propiedades antigénicas. Este enfoque contribuye al entendimiento de las dinámicas evolutivas del virus a nivel regional y mundial. Dicha comprensión fortalece la capacidad diagnóstica del país, aporta para la mejora en la bioseguridad y sanidad de la producción porcina nacional, y refuerza los planes de preparación frente a eventos zoonóticos potencialmente pandémicos.

La aproximación basada en secuencias empleadas en esta investigación representa una alternativa viable para la caracterización antigénica y selección de virus nacionales de campo que pueden ser utilizados tanto para la actualización de pruebas diagnósticas como para el posible desarrollo de biológicos contra la Influenza A en cerdos en el país. Estas metodologías favorecen los procesos de validación analítica y diagnóstica que deben aplicarse en la implementación de pruebas de laboratorio para el diagnóstico de enfermedades infecciosas como la influenza en porcinos, fortaleciendo así la independencia tecnológica y contriubyendo al desarrollo de nuevas estrategias en el control de la enfermedad en el país.

Mediante esta aproximación, se logró caracterizar 31 virus del subtipo H1N1 pandémico de 2009 (H1N1pdm), cuatro del subtipo H1N1 clásico y dos virus reordenados H1pdmN2 en cinco clústeres antigénicos. Esta agrupación permitió identificar seis virus de campo como potenciales antígenos de referencia nacional. Como parte de este proyecto, se estableció un banco de virus semilla antigénicamente estable para cada candidato. A partir

de estos virus semilla, se obtuvo un banco de antígenos que fue verificado mediante pruebas de Inhibición de la Hemaglutinación (HI), demostrando su capacidad de ser diferenciados entre sí en la mayoría de las ocasiones por sueros de porcinos del país.

Se evidenció que a través de la utilización de antígenos nativos, se mejoró el desempeño de la prueba de HI para la detección de anticuerpos específicos frente al FLUA virus y para la clasificación serológica de los individuos analizados, en comparación con el antígeno de referencia actualmente empleado para el virus H1. A través de asociaciones estadísticas observadas entre los candidatos, se logró reducir a tres el número de cepas potencialmente representativas. Cada una de estas representó uno de los subtipos detectados en Colombia (H1N1pdm, H1N1 clásico y H1pdmN2) y, de acuerdo con su rendimiento en la prueba de HI, fueron recomendados como nuevos antígenos a incluir en los protocolos serológicos en los laboratorios donde se realiza el diagnóstico.

En relación con el subtipo H3N2, este proyecto llevó a cabo la caracterización del único virus detectado hasta la fecha de este subtipo en Colombia. La metodología basada en secuencias reveló que el H3N2 porcino del país corresponde a una nueva variante genética y antigénica no reportada previamente. Adicionalmente fue posible cuantificar la divergencia entre el virus detectado en el campo y la cepa de referencia actualmente empleada para virus del subtipo H3N2 en el diagnóstico serológico de la enfermedad.

Como resultado de esta investigación, se generó un banco de virus semilla y un banco de antígenos, los cuales fueron verificados y comparados con antígenos de referencia internacionales adquiridos para la realización de la prueba. Se recomienda utilizar el virus H3N2 porcino colombiano como antígeno de referencia nacional, en la realización de la prueba HI, para lograr una mejor eficiencia de la prueba diagnóstica.

5.2 Recomendaciones

La recomendación principal que se desprende de esta investigación es la vigilancia continua de las características del virus de Influenza A en cerdos del país, tanto desde el punto de vista genético como antigénico. Como se demostró en este estudio, en el país

existe una considerable diversidad viral. El establecimiento de un programa de vigilancia de la Influenza en cerdos permitirá conocer la dinámica viral a nivel nacional, favoreciendo el desarrollo y la actualización de pruebas diagnósticas, así como la implementación de medidas de intervención y decisiones basadas en el análisis de datos genómicos pertinentes para el contexto colombiano.

Este estudio permitió la verificación y selección final de cuatro virus candidatos como referencia nacional para la prueba HI, sin embargo, se recomienda realizar un perfil antigénico de estos virus a través de técnicas *'gold standard'*. Además, debido a la variabilidad inherente del virus, se hace necesario evaluar periódicamente la representatividad de los virus propuestos y los sueros controles utilizados en la prueba y llevar a cabo las actualizaciones y ajustes necesarios conforme aparezcan nuevas variantes antigénicas en el país.