UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS DE LA PRODUCCIÓN Y DE LA SALUD ANIMAL

FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA

IDENTIFICACIÓN DE DIFERENCIAS GENÉTICAS Y SU CORRELACIÓN CON VARIACIONES ANTIGÉNICAS DE LOS SUBTIPOS DEL VIRUS DE INFLUENZA PORCINA QUE CIRCULAN EN EL TERRITORIO NACIONAL

T E S I S

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS DE LA PRODUCCIÓN Y DE LA SALUD ANIMAL

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Dedicatorias

Dedico este trabajo a:

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Resumen

La influenza porcina es un padecimiento a nivel mundial, que genera daños en el sistema respiratorio de los cerdos. En la población porcina de México circulan principalmente los subtipos H1N1 y H3N2. Existe evidencia de que nuevos subtipos del virus de influenza han evolucionado genéticamente y se han reordenado con virus humanos y de otras especies, debido a esto, nuestros objetivos fueron identificar y caracterizar los cambios genéticos que se han generado en los diferentes virus de influenza porcina en cerdos de la República Mexicana, así como las diferencias en la respuesta antigénica que genera cada uno de ellos. 486 muestras de pulmón de cerdo se procesaron por qRT-PCR: 10 (0.48%) fueron positivas y 476 (99.52) negativas. De las muestras positivas se logró secuenciar el genoma completo de tres subtipos H1N1, dos subtipos H3N2, un subtipo H1N2 y dos subtipos H5N2, de los cuales, un subtipo H1N1 tiene una alta relación genética con virus de influenza humanos, también se identificó un subtipo H1N2 relacionado con virus H1N2 porcinos reportados en Estados Unidos, así como dos subtipos H5N2 en los cuales los ocho segmentos son de pollo, siendo la primera ocasión que se reporta en cerdos de México. El análisis de estas secuencias nos ha permitido conocer la evolución genética de estos virus, demostrando que en la población porcina de México circulan virus que han sufrido reordenamientos de proteínas entre virus de diferentes subtipos, y que estos se han adaptado a la población porcina de México. Los resultados de variación antigénica mostraron que los virus aislados en el presente trabajo son antigénicamente diferentes entre ellos.

Palabras clave: Influenza porcina, Evolución de Influenzavirus, México.

Abstract

Swine influenza is a globally spread disease that causes respiratory damages in swine. In the swine population of Mexico mainly circulate H1N1 and H3N2 subtypes. There is evidence that new subtypes of the influenza virus have evolved genetically and have been rearranged with human and other species viruses, due to this, our objectives were to identify and characterize the genetic changes that have been generated in the different subtypes of the swine influenza viruses in pigs of the Mexican Republic, as well as the differences in the antigenic response generated between them. 486 samples of swine lung were processed by qRT-PCR: 10 (0.48%) were positive and 476 (99.52%) negative. From the positive samples, the complete genome of three H1N1 subtypes, two H3N2 subtypes and two H5N2 subtypes were sequence of which an H1N1 subtype has a high genetic relationship with human influenza viruses; also identified that a H1N2 subtype its related with H1N2 subtypes reported in the United States, as well as two H5N2 subtypes whose eight segments are chicken origin, being the first occasion reported in Mexico. The analysis of these sequences has allowed us to know the genetic evolution of these viruses, proving that in the swine population of Mexico are circulating viruses that suffered mutations and rearrangements on their proteins with different subtypes and that have adapted to swine porcine population of Mexico; also, with the results of antigenic variations we can conclude that the viruses isolated in the present work are antigenically different.

Keywords: Swine flu, Influenzavirus evolution, Mexico.

Declaración

El autor de ésta tesis otorga el consentimiento al Posgrado en Ciencias de la Producción y de la Salud Animal, de la Universidad Nacional Autónoma de México, para que ésta tesis se encuentre disponible para cualquier tipo de intercambio bibliotecario.

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José Manuel Saavedra Montañez

Lista de cuadros y figuras

Abreviaturas o siglas utilizadas

ARN	Ácido ribonucleico
ADN	Ácido desoxirribonucleico
cDNA	DNA complementario
IH	Inhibición de la hemoaglutinación
UHA	Unidades hemoaglutinantes
VIP	Virus de influenza porcina
HA	Hemoaglutinina
M1	Proteína de matriz
M2	Proteína M2 o canal de iones
NA	Neuraminidasa
NEP	Proteína de exporte nuclear
NP	Nucleoproteína
NS1	Proteína no estructural 1
PA	Polimerasa
PB1	Polimerasa básica 1
PB2	Polimerasa básica 2
ARNm	ARN mensajeros
RNP	Ribonucleoproteína
vRNPs	Ribonucleoproteínas virales
ARNv	ARN viral
EP	Embrión de pollo
SPF	Libre de patógenos específicos
DEPC	Dietilpirocarbonato
SA	Ácido siálico
ß-PL	ß-propiolactona
VP	Valores de protección
VRA	Valores de relación antigénica
PPA	Porcentaje de protección antigénica

1. INTRODUCCIÓN

1.1 Descripción del virus de la influenza

Los virus de influenza pertenecen a la familia *Ortomixoviridae*, se conocen cuatro tipos, clasificados como: Influenzavirus tipo A, B, C y D ó *Alfainfluenzavirus*, *Betainfluenzavirus*, *Gamainfluenzavirus* y *Deltainfluenzavirus* respectivamente de acuerdo a la última nomenclatura oficial publicada por el "International Committee on Taxonomy of Viruses" ("ICTV" 2018). Se clasifican en subtipos virales con base en la antigenicidad de las proteínas hemoaglutinina (HA) y neuraminidasa (NA) (Das et al. 2010). Actualmente se reconocen 18 tipos de HA y 11 tipos de NA (Tong et al. 2013). Los influenzavirus tipo A circulan en un gran rango de especies incluyendo a los humanos y animales domésticos: cerdos, caballos, aves de corral y aves migratorias silvestres (más de 100 especies de patos, gansos, cisnes, gaviotas y aves acuáticas silvestres son considerados como reservorios naturales), (Olsen et al. 2006; Yoon, Webby, and Webster 2014). Mientas que los tipos B y C afectan principalmente a humanos y los tipo D se ha reportado que infectan a vacas cabras y cerdos (Zhai et al. 2017) (Figura 1).

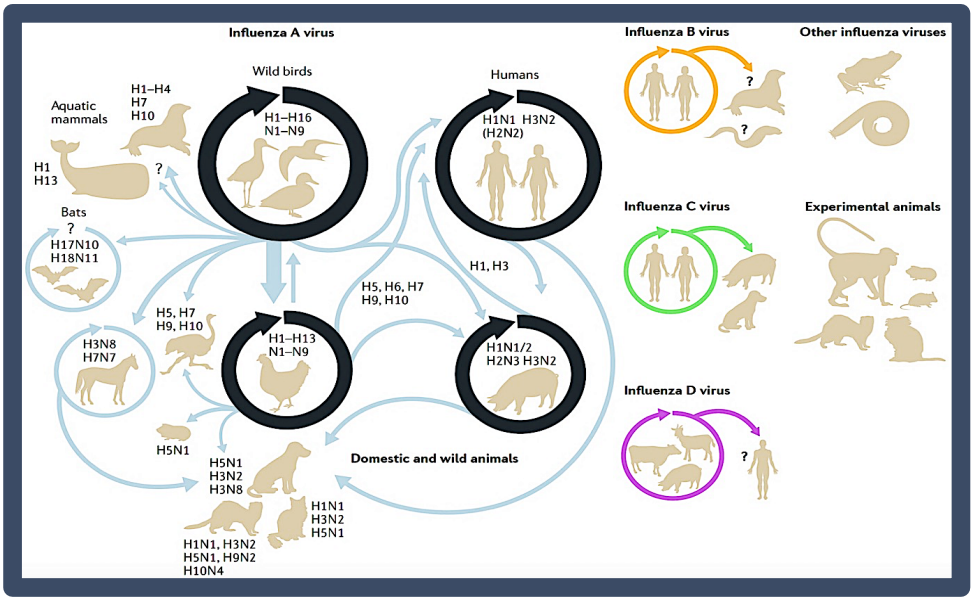

Figura 1. Subtipos y hospederos de los influenza virus. Modificado de: Jason, S. Long et al. Host and viral determinants of Influenza A virus species specificity, Nature 2018.

El genoma de los influenzavirus tipo A está integrado por ocho segmentos genéticos, el primer segmento codifica para la polimerasa básica 2. (PB2), el segundo para la polimerasa básica 1 (PB1), el tercero para la polimerasa ácida (PA), el cuarto para la hemoaglutinina (HA) que es responsable de la unión celular y la entrada a través de la unión de ácido siálico en la superficie de las células para la posterior fusión de la membrana endosomal con la envoltura viral, el quinto segmento codifica para la nucleoproteína del virus (NP); el sexto para la neuraminidasa (NA) que permite la liberación de nuevos viriones de células infectadas por la escisión de los enlaces entre HA y ácido siálico, facilitando la liberación del virus; el séptimo segmento (M) codifica para la proteína de la matriz (M1) y una proteína de la superficie del virus (M2) que actúa como un canal iónico; el octavo segmento (NS) codifica para la proteína no estructural 1 (NS1) que está involucrada en la evasión de la respuesta inmune y la exportación nuclear y para la proteína (NEP; también conocida como NS2), que participa en la exportación nuclear de complejos de ribonucleoproteínas virales. (Cohen et al. 2013) (Figura 2). También han sido descritas otras proteínas que han sido propuestas para inducir la muerte celular (PB1-F2) (Chen et al. 2001) y para modular la patogenicidad viral (PA-X) (Jagger et al. 2012).

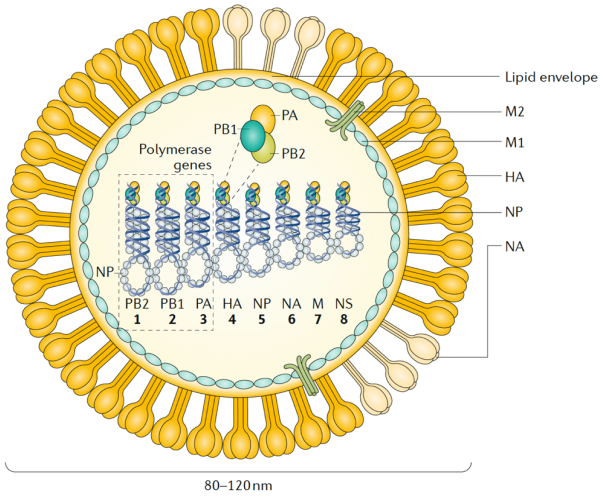

Figura 2. Estructura del virus de la influenza. Modificado de: Florian Kramer et al. Influenza. Nature Reviews. 2018.

1.2 Ciclo de replicación de los influenzavirus

La replicación del virus de influenza comienza con unión celular mediada por la unión de HA a los receptores de ácido siálico (SA) que se encuentran en la superficie de la célula. La entrada del virión es por endocitosis y la disminución del pH dentro del endosoma desencadena un cambio conformacional irreversible en la HA que expone el péptido de fusión y estimula la unión de la envoltura viral con la membrana endosomal (Bullough et al. 1994). Al mismo tiempo, el paso de protones e iones de potasio a través del canal iónico M2 acidifica el interior del virión y media la disociación de M1 y las ribonucleoproteínas virales (vRNPs), liberando las vRNPs en el citoplasma celular (Martin and Heleniust 1991; Stauffer et al. 2014), estas vRNPs son importadas al núcleo en donde ocurre la transcripción primaria (Engelhardt, Smith, and Fodor 2005). Los ARNm virales se exportan al citoplasma para su traducción por la maquinaria celular. Las recién sintetizadas polimerasas y nucleoproteínas se importan al núcleo para llevar a cabo la replicación y transcripción adicional (secundaria). Las nuevas vRNPs recién formadas son exportadas al citoplasma y transportadas a la superficie celular mediante M1 y NEP (Neumann, Hughes, and Kawaoka 2000) para el empaquetado y ensamblado con las proteínas estructurales (HA, NA, M1 y M2). La nueva progenie viral se forman al brotar de la membrana plasmática de la célula huésped y la posterior propagación es facilitada por NA que rompe el enlace entre el SA y la HA. (Palese, Tobita, and Ueda 1974) (Figura 3).

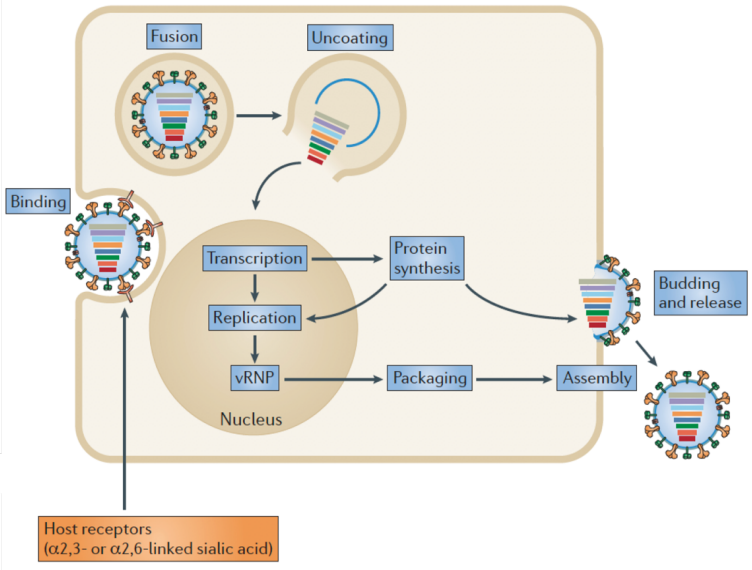

Figura 3. Replicación del virus de la influenza. Modificado de: Yi Shi et al, Enabling the 'host jump': structural determinants of receptor-binding specificity in influenza A viruses, Nature 2014.

1.3 Especificidad de los virus de influenza

La especificidad de los influenzavirus por su hospedador es explicado por la afinidad de unión al receptor (Figura 4). Los virus de influenza humana se unen preferentemente al ácido siálico que está vinculado a la galactosa por un enlace α-2,6-SA presente en las células epiteliales de la tráquea en humanos. En contraste los virus aviares preferentemente reconocen α-2,3-SA presente en células epiteliales del tracto intestinal de las aves (Rogers and Paulson 1983; Stevens et al. 2006), ésta especificidad al receptor sugiere que los virus de influenza necesiten adquirir la habilidad para reconocer a los receptores humanos como ocurrió en las pandemias de 1918, 1957 y 1968. Sin embargo, hay estudios que demuestran receptores de tipo aviar en células del tracto respiratorio en humanos (Shinya et al. 2006; Van Riel et al. 2006). Los cerdos pueden ser infectados de forma natural o experimental con virus aviar debido a que en las células epiteliales de la tráquea expresan receptores de tipo aviar y humano (Ito et al. 1998). Es por eso que los cerdos pueden ser infectados no sólo por virus humanos sino también por virus aviares (C. Scholtissek et al. 1983), es por

ésta razón que son considerados como un recipiente de mezcla (Castrucci et al. 1993; Christoph Scholtissek 1995).

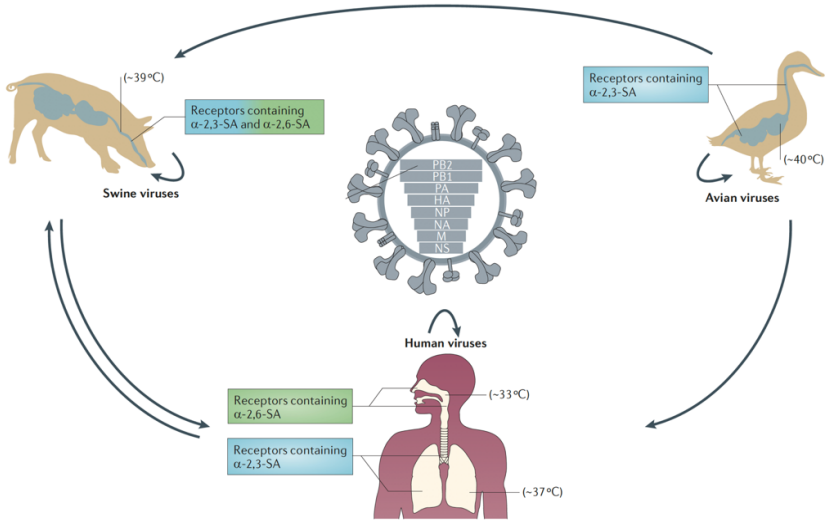

Figura 4. Receptores de los virus de influenza. Modificado de: Influenza A viruses: new research developments, García-Sastre, Nature 2011.

1.4 Mutaciones del virus de influenza

La evolución antigénica de los virus de influenza se produce a través de la deriva antigénica (del término en inglés: drift) caracterizada por la selección de nuevas cepas que contienen cambios de aminoácidos en la HA y la NA. Estos cambios superan parcialmente la inmunidad preexistente de la población y estas cepas son en gran parte responsables de las epidemias de influenza estacional (Webster et al. 1982; Zambon 1999; Carrat and Flahault 2007). Los cambios más dramáticos en los subtipos virales resultan del reordenamiento genético (del término en inglés: shift) que tradicionalmente se asocian con la aparición de virus pandémicos (Fields 2001). Estos ocurren cuando una célula hospedera es infectada por más de un subtipo diferente y los segmentos genómicos virales se reordenan y se desarrolla una nueva combinación (Carrat and Flahault 2007; Boni 2008) (Figura 5).

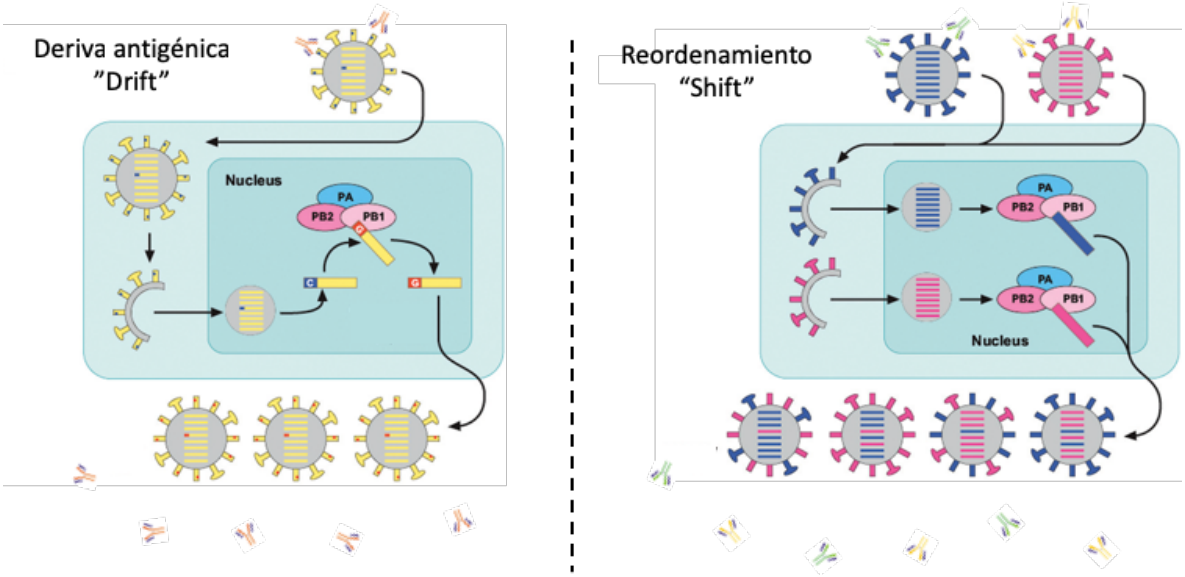

Figura 5. Tipos de mutaciones en los virus de influenza. Modificado de: Evasión of Influenza A Viruses from Innate and Adaptive Immune Responses, Carolien E. van de Sandt, Viruses 2012.

1.5 Cruce de barrera interespecie

Las infecciones en cerdos con influenzavirus humanos suelen ocurrir bajo situaciones naturales. Shope (1938) presentó evidencia serológica de que podía ocurrir, pero no fue hasta el aislamiento en Hong Kong del virus H3N2 de cerdos en Taiwán en 1970 (Kundin 1970), que las investigaciones comenzaron a examinar el potencial de transmisión de subtipos humanos a cerdos.

La influenza porcina no se reportó en Europa hasta 1976, cuando el virus clásico porcino H1N1 fue detectado en cerdos en Italia. Por la misma época, un virus totalmente humano H3N2 se presentó en la población porcina europea. En 1979, un virus de influenza aviar fue aislado de los cerdos en Italia, este virus H1N1 similar al aviar rápidamente sustituyó al clásico virus porcino H1N1 como el linaje dominante y se sometió a una redistribución con el virus H3N2 humano, dando lugar a un virus con HA y NA similar al humano y los genes internos similares al virus aviar (Campitelli et al. 1997).

Actualmente circulan tres subtipos principales de influenza en la población porcina alrededor del mundo H1N1, H3N2 y H1N2, aunque antes de 1998 los virus de influenza en cerdos de Estados Unidos habían sido casi exclusivamente el subtipo H1N1 clásico (Chambers et al. 1991). Un aumento en la diversidad genética y antigénica coincide con la aparición de la triple recombinante H3N2 en 1998, así como la combinación de los genes PA y PB2 aviares y el gen PB1 humano lo que parece aumentar el ritmo de la deriva antigénica y su redistribución con lo que se aumenta la capacidad viral para evadir la respuesta inmune (Vincent et al. 2008).

A finales de 1998 dos virus de influenza H3N2 diferentes fueron aislados de cerdos que presentaban enfermedad severa, similar a la influenza en Carolina del Norte, Minnesota, Iowa y Texas. El aislamiento de Carolina del Norte se identificó como un virus rearreglante que contenía HA, NA y PB1 similares al virus contemporáneo de influenza humana y los genes M, NP, NS, PA y PB2 similares al linaje del virus clásico de influenza porcina H1N1. Los aislamientos de Minnesota, Iowa y Texas (triple rearreglante) fueron aún más complejos; al igual que el aislamiento de Carolina del Norte, estos virus contenían HA, NA y PB1 de un linaje del virus humano contemporáneo y M, NP y NS del linaje clásico porcino H1N1. Sin embargo, estas cepas contenían genes PA y PB2 de un linaje de virus de la influenza aviar. Después de la aparición de estos dos virus rearreglantes, el doble rearreglante no continuó circulando, pero el virus triple rearreglante se estableció en la población porcina y siguió circulando y evolucionando (Zhou et al. 1999).

La vigilancia virológica prospectiva realizada entre marzo de 1998 y junio del 2000 en Hong Kong, sobre los cerdos importados del sureste de China, proporcionó la primera evidencia de transmisión entre especies del virus de influenza aviar H9N2 a los cerdos y documentó su co-circulación con los virus humanos contemporáneos H3N2 (A/Sydney/5/97, Sydney97). Todos los segmentos del genoma del virus H9N2 porcino estaban estrechamente relacionados con virus similares a gallina/Beijing/1/94 (H9N2), pato/Hong Kong/Y280/97 (H9N2), y los descendientes del linaje de este último virus. El análisis filogenético sugiere que la repetida transmisión entre especies, ocurrió desde el huésped aviar a los cerdos. Los virus Sydney97 (H3N2) aislados de cerdos estaban

estrechamente relacionadas con el virus humano contemporáneo H3N2 en todos los segmentos de genes y no había sido objeto de redistribución génica (Peiris et al. 2001).

En octubre de 1999, un virus de influenza A H4N6 fue aislado de cerdos con neumonía en una granja porcina comercial en Canadá. Los análisis filogenéticos de las secuencias de los ocho segmentos de ARN viral, demostró que estos eran en su totalidad virus aviar del linaje de América del Norte (Karasin et al. 2000). En el 2004, un nuevo virus rearreglante H3N1, fue identificado de cerdos que presentaban tos en Estados Unidos. El análisis filogenético de la secuencia de nucleótidos demostró un segmento HA con 95,9 a 99,5% de similitud en los nucleótidos con respecto a la parte III del virus de influenza porcino H3N2, que es el genotipo predominante H3 que circula entre los cerdos en EE.UU. El segmento NA era muy similar al del virus H1N1 clásico, con 92 a 93% de identidad entre las secuencias virales disponibles en el GenBank, pero mostraron una mayor homología (98 a 99%) con el virus H1N1 contemporánea del Medio Oeste. Otros genes eran de origen porcino (M, NP y NS1), aviar (PA y PB1) y humano (PB2), representante de la composición interna del gen contemporánea del virus porcino triple rearreglante H3N2 de América del Norte. Esto sugiere que los virus de la influenza H3N1 A/swine/Minnesota/00395/2004 son un reordenamiento que contiene genes del virus de influenza porcina triple rearreglante H3N2 y del virus de influenza porcino contemporáneo H1N1 (W. Ma et al. 2006).

En septiembre del 2006, un virus de influenza A/swine/Missouri/4296424/2006 (Sw/4296424) fue aislado de varios cerdos de 5 a 6 semanas de edad que presentaban bronconeumonía multifocal. Estos virus fueron identificados a través de la base de datos de GenBank como virus de influenza H2N3. Según el análisis filogenético el gen HA del virus Sw/4296424 coincide más estrechamente con los virus H2 aislados de patos en el norte de América (hasta el 97,8% de identidad en la secuencia de nucleótidos). El segmento NA estaba estrechamente relacionado con el virus de la gripe aviar H4N3 aislado de cerceta de alas azules (identidad 98,3%). Con la excepción del gen PA, los genes internos fueron derivados de los virus porcinos contemporáneos triples rearreglantes que se encuentra actualmente en Estados Unidos (Wenjun Ma et al. 2007).

Durante la vigilancia del virus de influenza porcina entre el 2004 y el 2006, dos cepas del virus de la influenza H3N8 fueron aislados de cerdos en China central. La secuenciación y el análisis filogenético de los ocho segmentos de genes revelaron que los dos aislamientos fueron de origen equino y más relacionado con el virus europeo de influenza equina de la década de los noventa. Una comparación de la secuencia de aminoácidos de la hemoaglutinina demostró varias sustituciones importantes. Esta expansión de la gama de huéspedes de los virus de la influenza equina H3N8 con mutaciones en la proteína HA podría plantear la posibilidad de transmisión de estos virus a los seres humanos (Tu et al. 2009).

En el 2006, Shin y colaboradores, reportaron el aislamiento de un virus de influenza H3N1 en cerdos que presentaron una infección respiratoria en dos granjas comerciales en Corea. El análisis filogenético demostró que los genes HA del virus porcino de Corea H3N1 se encontraba colocado en un fragmento diferente que los otros virus porcinos H3. Sin embargo, comparten la misma raíz con los grupos II y III de los virus porcinos H3N2 encontrados en Estados Unidos. El gen NA está colocado en el "cluster" porcino de Estados Unidos y está más relacionado con el virus Sw/Wisconsin/238/97, que es un virus aislado en Estado Unidos. Los genes restantes están más estrechamente relacionados con los virus Coreanos H1N1 y H1N2, esto sugiere que el virus H3N1 es un reordenamiento entre un linaje desconocido del virus H3N2 y el virus de influenza porcina (Shin et al. 2006).

A principios del 2008, dos virus porcinos H5N2 fueron aislados en Corea. La secuenciación y análisis filogenético de las proteínas de superficie revelaron que los virus Sw/Corea/C13/08 y Sw/Corea/C12/08, fueron derivados de virus de influenza aviar del linaje euroasiático. Sin embargo, aunque el virus Sw/Korea/C12/08 es un virus enteramente aviar, el virus aislado Sw/Korea/C13/08 es un reordenamiento entre virus aviar y porcino, con los genes PB2, PA, NP y M procedente del virus porcino Coreano del 2006, H3N1 (Lee et al. 2009).

Hasta la fecha, Asia sigue siendo la única región en la que co-circulan en los cerdos linajes conocidos de América del norte y Eurasia. Sin embargo, los análisis evolutivos de virus

H1N1pdm09 humanos en Asia no son compatibles con un origen asiático de la pandemia de 2009 (Lemey, Suchard, and Rambault 2009). Debido a la necesidad de una mejor comprensión evolutiva de la influenza, se han desarrollado protocolos que hacen que sea posible aplicar técnicas de secuenciación a gran escala para el genoma de la influenza (Ghedin et al. 2005). Pero a pesar del gran aumento de secuenciación de genes del virus de influenza que ha tenido lugar en los últimos años, la respuesta a cuándo, dónde, y cómo surgió el virus pandémico H1N1pdm09 sigue siendo desconocido, ya que la gran mayoría de las secuencias disponibles públicamente son de virus humanos y aviares, y no a partir de cepas porcinas. En México un estudio de caracterización genética de un aislado porcino, identificó a este virus como un virus similar al pandémico de 2009 y los cuales co-circularon en cerdos durante la pandemia. En este mismo estudio a partir de aislamientos de La Gloria durante el brote de 2009 indicó que este virus está estrechamente relacionado con cepas del mismo tiempo aisladas en California (Escalera-Zamudio et al. 2012).

2. JUSTIFICACIÓN

La pandemia generada por un subtipo H1N1 en humanos en 2009 pudo ser predicha si se tuvieran datos de virus de influenza presentes en otras especies susceptibles, como aves y cerdos. Por lo que es necesario establecer políticas de vigilancia epidemiológica, montar técnicas de diagnóstico específicas y sensibles, así como tener ceparios actualizados de los virus para evaluar su evolución y distribución en la República Mexicana, además de calcular el riesgo que esta evolución implica. Por lo tanto, es de interés aislar y caracterizar los influenzavirus que circulan en la población porcina mexicana, y comparar sus secuencias genómicas con otros virus aislados en todo el mundo, incluyendo a los virus estacionales y pandémicos en humanos, cerdos y otras especies.

3. HIPÓTESIS

En la población porcina de México circulan nuevos subtipos del virus de influenza que han evolucionado genéticamente y se han reordenado con virus humanos y de otras especies, siendo antigénicamente diferentes entre ellos.

4. OBJETIVO GENERAL

Evaluar las características genéticas presentes en los virus de influenza porcina en México y sus características antigénicas que genera cada uno de los subtipos.

4.1 Objetivos específicos

1. Realizar aislamientos del virus de influenza porcina en cerdos sacrificados en rastros y unidades de producción de diferentes estados de la República Mexicana.
2. Identificar la presencia y distribución de nuevos subtipos del virus de influenza porcina.
3. Evaluar los cambios genéticos de nuevos subtipos del virus de influenza porcina.
4. Determinar la variación antigénica de los nuevos subtipos del virus de influenza porcina.

5. MATERIAL Y MÉTODOS

5.1 Obtención de muestras

La investigación se llevó a cabo con muestras del tracto respiratorio (pulmón, tráquea y linfonodos) de cerdos provenientes de unidades de producción de la República Mexicana. Se estableció un acuerdo para la obtención de muestras de cerdos a nivel de rastro, la cantidad de muestras requeridas dependió del número de estados de donde provienen los animales. Todos los tejidos fueron conservados a -70°C hasta el momento de analizarlos. Se observan pulmones y cortes de tejidos (pulmón, tráquea y linfonodo) (Figura 6).

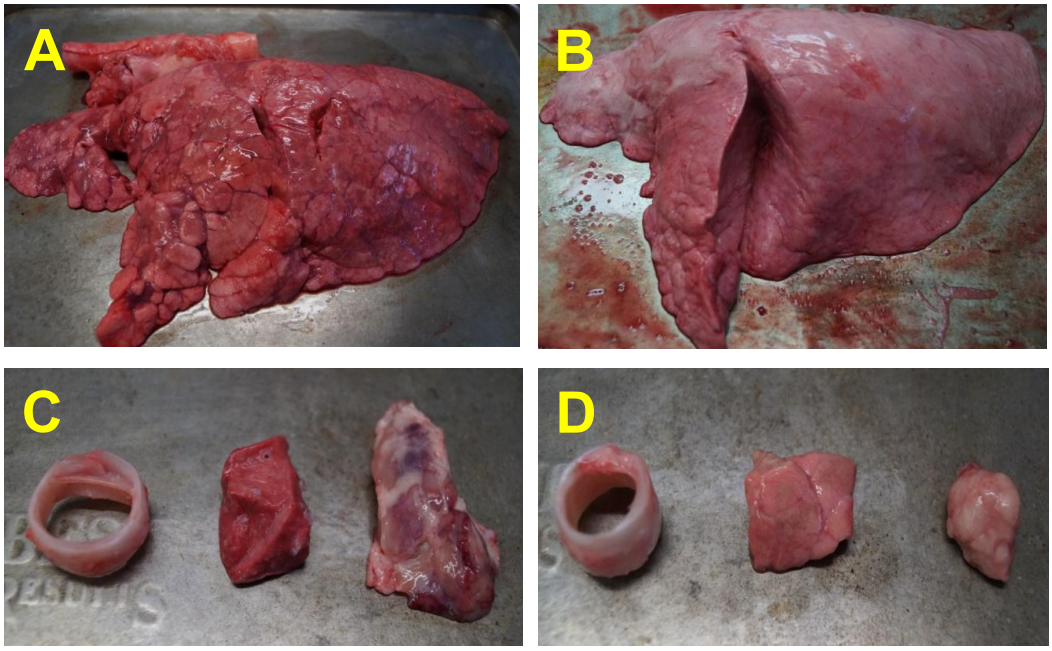

Figura 6. Tejidos que se emplearon. (A) Pulmón con lesiones; (B) Pulmón sin lesiones evidentes; (C) Cortes de tejidos con lesiones, de izquierda a derecha tráquea, pulmón y linfonodo mediastínico; (D) Cortes de tejidos sin lesiones evidentes, de izquierda a derecha tráquea, pulmón y linfonodo mediastínico.

Por otra parte se estableció un acuerdo de colaboración con una empresa de productos biológicos para que nos enviarán muestras del tracto respiratorio de cerdos de diferentes estados de la República Mexicana con signos clínicos sugestivos a influenza porcina (fiebre, escurrimiento nasal, estornudos, disnea), para ello se diseñó una guía práctica que

se distribuyó a los médicos veterinarios responsables de tomar las muestras para la correcta colección y envío de estas.

Los estados de los que se obtuvieron las muestras fueron: Jalisco, Estado de México, Guanajuato, Sonora, Michoacán, Puebla, Morelos, Yucatán, Querétaro, Hidalgo, Veracruz y Nuevo León. El estado del que mayor cantidad de muestras se obtuvieron fue Jalisco con 225 (46.30%) y del que se obtuvieron la menor cantidad de muestras fue Nuevo León con 2 (0.41%) (Figura 7).

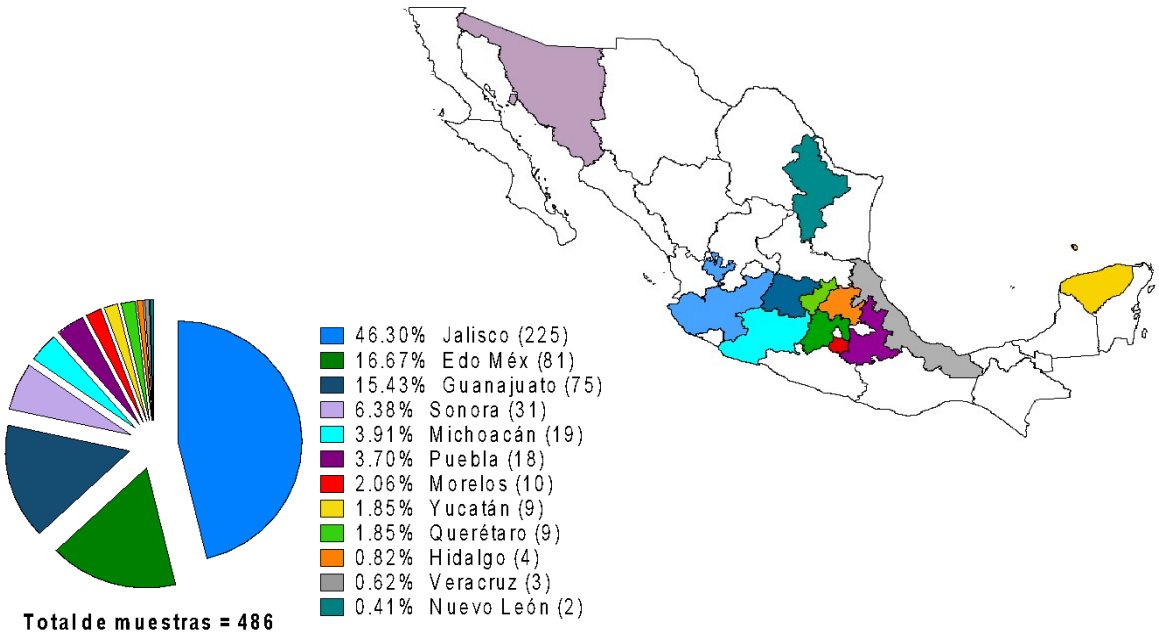

Figura 7. Porcentaje y número de muestras por Estado. En el mapa se observan en distinto color los Estados muestreados y en la gráfica se observa el porcentaje y número de muestras obtenidas en cada Estado.

5.2 qRT-PCR

El sistema de amplificación consiste en un sistema basado en Transcripción Reversa (RT) y Reacción en Cadena de la Polimerasa en Tiempo Real (qRT-PCR), para detectar y cuantificar la presencia del genoma del virus de Influenza tipo A, empleando como blanco de amplificación el gen M de este virus (que codifica para la proteína de la matriz). El sistema emplea ARN obtenido de tejidos, iniciadores y sondas TaqMan, así como los reactivos que permiten realizar la transcripción reversa y la qRT-PCR. Este sistema resulta

extremadamente seguro, gracias a la incorporación de un control interno que permite monitorear la presencia de sustancias inhibitorias y descartar falsos negativos. El sistema también incluye un ADN control positivo, que permite construir una curva de referencia para cuantificar el número de copias virales presentes en la muestra.

5.3 Aislamiento viral

Para aislar y propagar los diferentes subtipos del virus de influenza, se utilizaron los macerados de los tejidos que resultaron positivos por qRT-PCR para generar inóculos, los cuales se filtraron (0.22 micras) y se inocularon en embriones de pollo (EP) libres de patógenos específicos (SPF) de 9-11 días de edad. A las 72 hrs post-inoculación los líquidos alantoideos se colectaron y se probaron mediante la prueba de hemoaglutinación.

5.4 Replicación viral en embrión de pollo

La propagación viral se realizó con los 5 aislamientos del virus de influenza, previamente filtrados (0.22 micras), se inocularon en EP-SPF de entre 9-11 días de edad, cada embrión fue inoculado en la cavidad alantoidea con 200 µl. Los embriones se incubaron a 37ºC, se cosecharon los líquidos alantoideos a las 24, 48 y 72 hrs post-inoculación y se titularon por la prueba de hemoaglutinación.

5.5 Titulación viral por hemoaglutinación

El líquido alantoideo de los embriones inoculados se tituló por medio de hemoaglutinación, en diluciones dobles seriadas iniciando 1:2 y empleando eritrocitos de pollo al 0.5%, esta prueba determina la presencia de virus con capacidad hemoaglutinante. Los procedimientos de propagación y titulación de los virus se realizaron en el laboratorio de bioseguridad del Departamento de Medicina y Zootecnia de Cerdos FMVZ-UNAM.

5.6 Secuenciación

De los casos positivos detectados por qRT-PCR se procedió a la secuenciación masiva de los ocho segmentos del virus con el equipo Ion Torrent de la Unidad de Investigación de la FMVZ, el análisis de las secuencias nos permitió determinar las diferencias genéticas que se han generado en los influenzavirus. También se realizaron análisis filogenéticos que nos permitieron conocer la evolución genética de dichos subtipos virales.

5.7 Análisis filogenético

Las secuencias obtenidas, fueron comparadas con las reportadas en el GenBank, seleccionando aquellas con un porcentaje de identidad superior al 95%, usando BLAST (Basic Local Alignment Search Tool) del NCBI. El análisis filogenético fue elaborado de forma independiente para cada segmento genómico con el programa MEGA 7.0.26, en la construcción filogenética se empleó el método de Maximum Likelihood con un modelo de sustitución General Time Reversible (GTR) y una distribución gama con sitios invariables (Nei M and Kumar S, 2000; Kumar S, Stecher G and Tamura K, 2016). La edición de los árboles se realizó con el programa FigTree.

5.8 Inactivación de virus con ß-propiolactona

En el DMZC se han realizado diversos trabajos inactivando virus de influenza con algunos métodos tales como luz UV, temperatura, rayos gamma, formalina y β-propiolactona (β-PL), obteniendo resultados favorables (Juárez, 2013; Mora Díaz, 2014). En el presente trabajo se inactivaron los virus con β-PL. Los lotes virales se centrifugaron a 5000 rpm (rotor eppendorf 16ª4.44 4x250 g) por 10 minutos para eliminar precipitados, posteriormente se filtraron (0.22 micras). Se agregó a cada subtipo viral β-PL al 98% en una dilución 1:300 de manera lenta (por goteo y en agitación), se dejaron agitando a 4ºC y después de 24 horas se conservaron a 4ºC hasta su posterior empleo.

5.9 Prueba de inactivación viral

La inactivación se verificó mediante la inoculación de virus inactivado con β-PL en embriones de pollo SPF de 11 días de edad, se revisaron los embriones cada 24 hrs durante 3 días para ver que no murieran, al finalizar la prueba se colectó el líquido y se tituló por hemoaglutinación.

5.10 Purificación de los virus de influenza

Se purificaron los virus inactivados de influenza por gradientes discontinuos de sacarosa, como se describe a continuación:

1. Los concentrados virales se clarificaron por centrifugación a 5000 rpm por 15 minutos (rotor eppendorf 16ª4.44 4x250 g).
2. Posteriormente los sobrenadantes se depositaron sobre colchones de sacarosa al 10%, 22% y 40% diluida en buffer NT (NaCl 0.5 M y Tris 0.05M, pH 7.5).
3. Se ultra centrifugaron a 31,000 rpm (rotor Beckman LE-80K Type 70Ti) durante 2 horas.
4. Se colectó la interfase 18%-22 de sacarosa, esta se diluyó con 20 ml de buffer NT.
5. Posteriormente se ultracentrífuga a 35,000 rpm (rotor Beckman LE-80K Type 70Ti) durante 1 hora.
6. Finalmente se decantó el sobrenadante que contiene restos de sacarosa y el botón de virus se resuspendió en 200 μl de buffer NT con 0.08% de azida de sodio y se conservaron a -70ºC hasta su uso.

5.11 Adición del adyuvante

Para preparar las dosis de inmunización, el concentrado viral purificado fue mezclado con aceite de cacahuate, aceite mineral, span y solución salina para potenciar su

inmunogenicidad. La proporción de los componentes del adyuvante es la siguiente: aceite de cacahuate 25%, aceite mineral 20%, span 5% y solución salina 50%.

5.12 Modelo de inoculación para generar sueros hiperinmunes

Se adquirieron 20 cerdos de 21 días de edad de una granja comercial. Los cerdos se mantuvieron bajo resguardo en unidades de aislamiento experimental en el Centro Nacional de Investigación Disciplinaria en Microbiología Animal (CENID-Microbiología). Todos los cerdos fueron alimentados bajo una dieta comercial, teniendo acceso al agua *ad libitum* y se les proporcionó enriquecimiento ambiental para su bienestar animal. Al momento de llegar se les hizo la prueba de inhibición de la hemoaglutinación (IH) para saber que eran libres a influenzavirus, considerando positivos a aquellos que tiene un título superior a 1:80, y de acuerdo a los resultados se descartaron dos cerdos con título de 1:80 y uno con título de 1:40. Después de tres días de adaptación (24 días de edad), los cerdos fueron distribuidos en cinco grupos experimentales los cuales se inocularon con los virus inactivados por vía intramuscular en la región lateral del cuello (cuadro 1).

Cuadro 1. Distribución de los cerdos en grupos experimentales.

Grupos	Influenzavirus mexicanos
G1 (n = 3)	A/swine/Mexico/Mich40/2010(H3N2)[a] **Mich40**[b]
G2 (n = 3)	A/swine/Mexico/EdoMexDMZC03/2015(H5N2)[a] **EdoMexDMZC03**[b]
G3 (n = 3)	A/swine/Mexico/GtoDMZC01/2014(H1N2)[a] **GtoDMZC**[b]
G4 (n = 3)	A/swine/Mexico/GtoDMZC04/2015(H1N1)[a] **GtoDMZC04**[b]
G5 (n = 3)	A/swine/Mexico/JalDMZC05/2015(H1N1)[a] **JalDMZC05**[b]
Mock (n = 2)	-

En la columna izquierda se observa la distribución de los grupos de cerdos y en la columna derecha los virus con los que se inocularon; [a] = Clasificación internacional de los influenzavirus; [b] = Abreviación utilizada en el presente trabajo.

5.13 Prueba de inhibición de la hemoaglutinación en cerdos

El día en que llegaron los cerdos a las instalaciones se les identificó con arete y se les tomó una muestra de sangre a través de la vena yugular, se separó el suero y fue inactivado a 56ºC durante 30 minutos, posteriormente se absorbieron con caolín y eritrocitos de ave al 5%, finalmente los sueros se centrifugaron a 2000 rpm (rotor eppendorf 16ª4.44 4x250 g) durante 10 minutos para realizar la técnica de IH, empleando el método establecido por la Organización de Salud Animal (OIE) (World Organisation for Animal Health, 2015), con las siguientes modificaciones: los virus se ajustaron a ocho unidades hemoaglutinantes (UHA), se realizaron diluciones dobles seriadas iniciando 1:5 hasta 1:2560, los sueros se consideraron positivos con títulos ≥1:80.

5.14 Índice de variación antigénica

Para cada uno de los aislamientos el porcentaje de protección obtenido con los desafíos de virus homólogos y heterólogos fue calculado usando la ecuación de Archetti and Horsfall (Archetti Italo, 1950). Los valores de protección (VP) fueron calculados como se describe a continuación: Los títulos de IH son usados en el cálculo de los valores de relación antigénica (VRA). Los títulos fueron usados para establecer los valores de r1 y r2, en donde r1= título heterólogo #2/título homólogo #1, y r2 = título heterólogo/homólogo #2. Los porcentajes de protección antigénica (PPA) que tengan un valor de 0 fueron reemplazados con el valor 0.01 para poder realizar los cálculos mediante la multiplicación de los valores de r (r1 x r2), del cual el resultado se multiplica por 100 para convertirlo en porcentaje.

6. RESULTADOS

Se procesaron 486 muestras de pulmón de cerdo, de estas 10 (0.48%) fueron positivas y 476 (99.52) negativas por qRT-PCR, de las muestras positivas se logró secuenciar el genoma completo de tres subtipos H1N1 de los estados (2 de Guanajuato y 1 de Jalisco), dos subtipos H3N2 provenientes de los estados (1 de Jalisco y 1 de Hidalgo), una secuencia

H1N2 del estado de Guanajuato; y dos secuencias H5N2 de los estados (1 de Guanajuato y 1 del Estado de México) (Cuadro 2).

Cuadro 2. Número de positivos y negativos de acuerdo al origen y secuencias obtenidas en este estudio.

	ESTADO	(-)	(+)	SECUENCIAS OBTENIDAS	IDENTIFICACIÓN EN ARBOLES*
RASTRO					
	Jalisco	207	1	A/swine/Mexico/JalDMZC12/2015(H3N2)[a] MH006723-MH006730[b]	JalDMZC12H3N2_swine_MEX_2015.
	Estado de México	78	0	-	
	Guanajuato	60	1	A/swine/Mexico/GtoDMZC09/2015(H1N1)[a] MH006699-MH006706[b]	GtoDMZC09H1N1_swine_MEX_2015.
	Michoacán	3	1	-	
	Hidalgo	2	1	A/swine/Mexico/HgoDMZC11/2015(H3N2)[a] MH006715-MH006722[b]	HgoDMZC11H3N2_swine_MEX_2015.
	Puebla	3	0	-	
GRANJA					
	Sonora	30	1	-	
	Jalisco	16	1	A/swine/Mexico/JalDMZC05/2015(H1N1)[a] MH013200-MH013207[b]	JalDMZC05H1N1_swine_MEX_2015.
	Michoacán	15	0	-	
	Puebla	15	0	-	
	Guanajuato	11	3	A/swine/Mexico/GtoDMZC04/2015(H1N1)[a] MH013192-MH013199[b]	GtoDMZC04H1N1_swine_MEX_2015.
					GtoDMZC01H1N2_swine_MEX_2014.
				A/swine/Mexico/GtoDMZC01/2014(H1N2) [a] KT225468-KT225475[b]	GtoDMZC02H5N2_swine_MEX_2014.
				A/swine/Mexico/GtoDMZC02/2014(H5N2)[a] KU141369-KU141376[b]	
	Morelos	10	0	-	
	Querétaro	9	0	-	
	Yucatán	9	0	-	
	Veracruz	3	0	-	
	Estado de México	2	1	A/swine/Mexico/EdoMexDMZC03/2015(H5N2)[a] MH013208-MH013215[b]	EMXDMZC03H5N2_swine_MEX_2015.
	Nuevo León	2	0	-	
	Hidalgo	1	0	-	
TOTAL		476	10	8	

* = Identificación empleada en la construcción de los arboles filogenéticos inferidos; [a] = clasificación internacional de los virus de los que se obtuvo la secuencia de su genoma; [b] = número de acceso al GenBank de las secuencias de los virus que se secuencio su genoma.

6.1 Análisis filogenético de los genes de HA y NA

Con respecto a la estructura evolutiva de las topologías inferidas del gen HA, el análisis de las secuencias nos permitió identificar que la proteína HA del virus GtoDMZC01/2014(H1N2) se encuentra en un clado con influenzavirus porcinos y humanos

H1N2 reportados en Estados Unidos en el periodo 2005-2010. Mientras que la HA de los virus GtoDMZC02/2014(H5N2) y el EdoMexDMZC03/2015(H5N2) se encuentran en un clado de influenzavirus aviares H5N2 aislados en México en 1994 y 1995, la HA del virus GtoDMZC04/2015(H1N1) se encuentra en un clado de influenzavirus humanos H1N1 aislados en Estados Unidos en 2013 y con uno de México aislado en 2014. Por otra parte, existe una homología entre las HA de los JalDMZC05/2015(H1N1) y GtoDMZC09/2015(H1N1) y se encuentran en un clado de influenzavirus porcinos H1N1 aislados en México en el periodo 2012-2014. La HA del virus HgoDMZC11/2015(H3N2) se encuentra en un clado de influenzavirus porcinos de México H3N2 aislados en 2012 y 2013. La HA del virus JalDMZC12/2015(H3N2) se encuentra en un clado con influenzavirus porcinos de Canadá y Estados unidos aislados en 2005 y 2006 (Figura 8). Con respecto a la estructura evolutiva de las topologías inferidas del gen NA, el análisis de las secuencias nos permitió identificar que las proteínas NA de los virus GtoDMZC01/2014(H1N2) y HgoDMZC11/2015(H3N2) se encuentran en un clado con influenzavirus porcinos de México H3N2 aislados en 2012 y 2013. Mientras que la NA de los virus GtoDMZC02/2014(H5N2) y el EdoMexDMZC03/2015(H5N2) se encuentran en un clado de influenzavirus aviares H5N2 aislados en México en 1994 y 1995 y con influenzavirus aislados en Estados Unidos en 1994 y 1995. La NA del virus GtoDMZC04/2015(H1N1) se encuentra en un clado con influenzavirus humanos H1N1 aislados en Estados Unidos en 2013 y 2014. El análisis de las secuencias nos permitió identificar que la proteína NA de los virus JalDMZC05/2015(H1N1) y el GtoDMZC09/2015 (H1N1) se encuentran en un clado de influenzavirus porcinos H1N1 aislados en México en el periodo 2012-2014. La NA del virus JalDMZC12/2015(H3N2) se encuentra en un clado con influenzavirus porcinos de Canadá y Estados Unidos aislados en 2005 y 2006 (Figura 9).

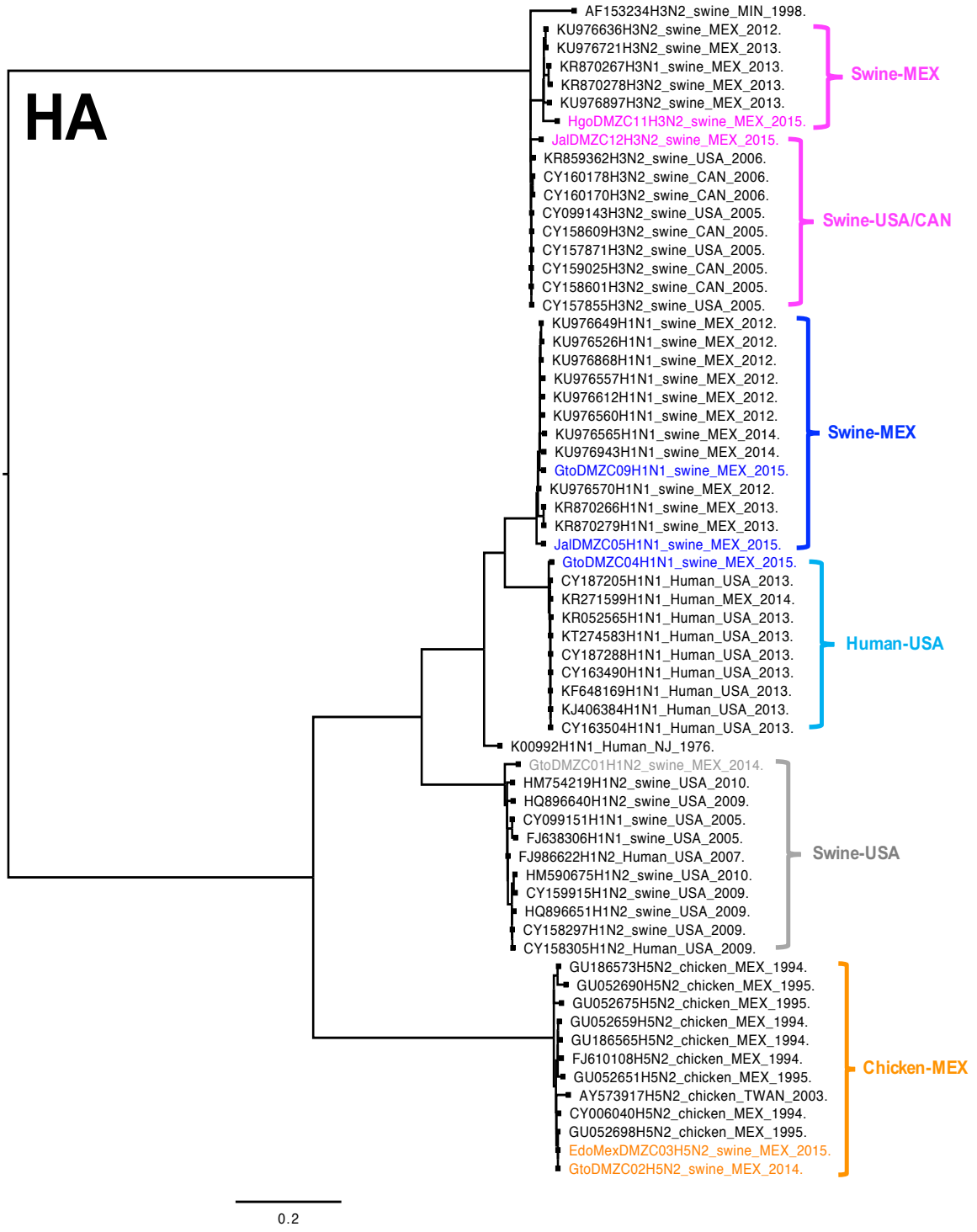

Figura 8. Árbol filogenético de la HA. En la parte inferior se observa la escala de las longitudes de las ramas, las cuales corresponden al número de sustituciones por sitio. Las secuencias de los influenzavirus mexicanos son observado en color según el subtipo: H1N1 (azul), H3N2 (rosa), H1N2 (gris), H5N2 (naranja).

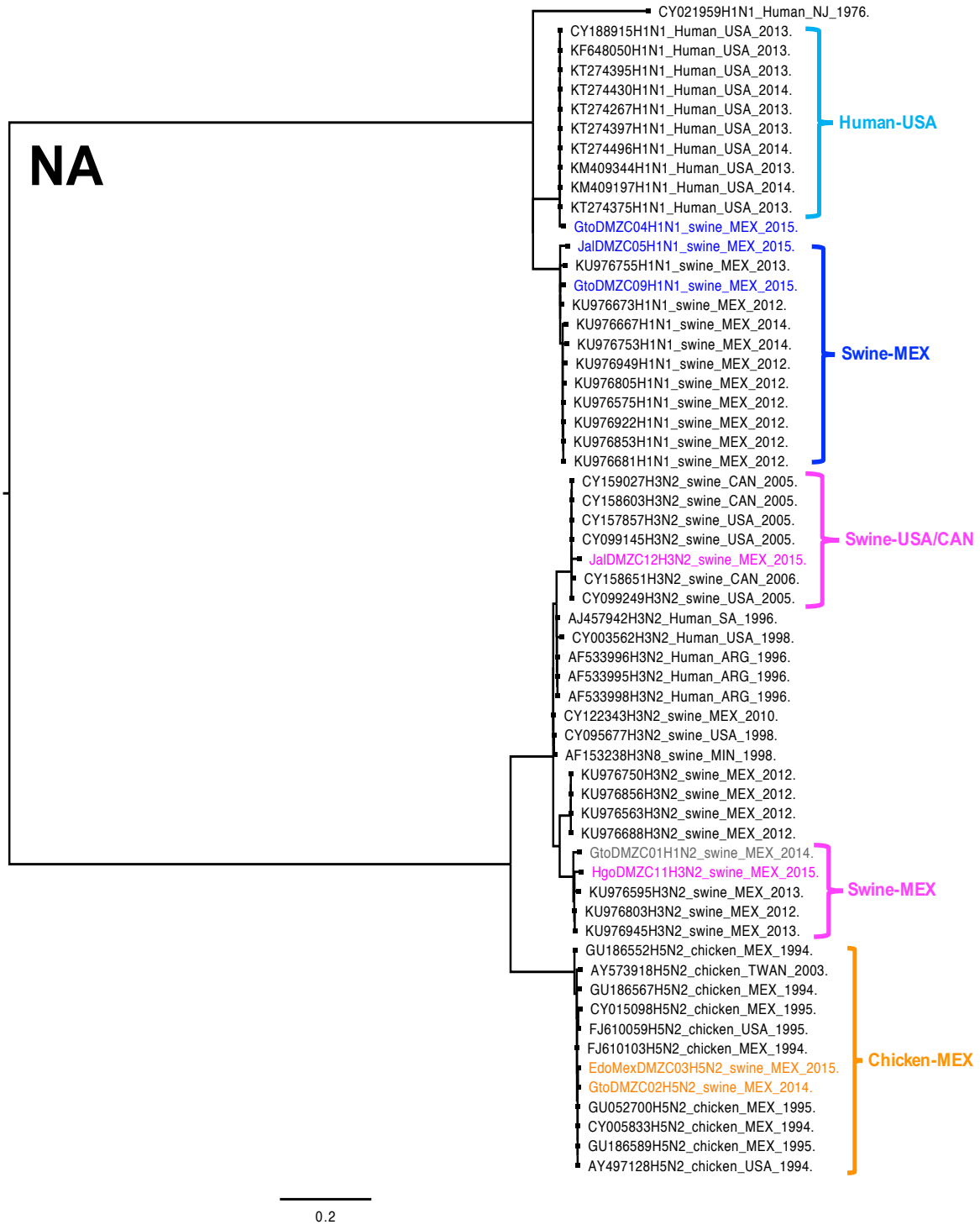

Figura 9. Árbol filogenético de la NA. En la parte inferior se observa la escala de las longitudes de las ramas, las cuales corresponden al número de sustituciones por sitio. Las secuencias de los influenzavirus mexicanos son observado en color según el subtipo: H1N1 (azul), H3N2 (rosa), H1N2 (gris), H5N2 (naranja).

Con respecto a la identificación del origen de los ocho segmentos genéticos de los virus de influenza aislados en México, se muestra que los ocho segmentos que integran al virus GtoDMZC01/2014(H1N2) son de origen porcino, los genes de las proteínas PB1, PA, NP y NS corresponden al subtipo H1N1, la NA al subtipo H3N2 y las proteínas PB2, HA y M al subtipo H1N2. En el caso de los virus GtoDMZC02/2014(H5N2) y EdoMexDMZC03/2015(H5N2) sus ocho segmentos son de origen aviar y corresponden al subtipo H5N2. Por otra parte, los ocho segmentos del virus GtoDMZC04/2015(H1N1) son de origen humano y corresponden al subtipo H1N1, mientras que los ocho segmentos del virus JalDMZC05/2015(H1N1) son de origen porcino y también corresponden al subtipo H1N1. Los ocho segmentos del virus GtoDMZC09/2015(H1N1) son de origen porcino y las proteínas PB2, PB1, PA, HA, NP, NA y M corresponden al subtipo H1N1; sin embargo, NS pertenece al subtipo H3N2. Cuando observamos el virus MichDMZC10/2015(H1N1) sus ocho segmentos son de origen humano y las proteínas PB2, PA, HA, NP, NA y M corresponden al subtipo H1N1 y las proteínas PB1 y NS al subtipo H3N2. Los ocho segmentos HgoDMZC11/2015(H3N2) son de origen porcino y las proteínas PB1, NP, M y NS corresponden al subtipo H1N1 y las proteínas PB2, PA, HA y NA al subtipo H3N2. Finalmente, los ocho segmentos del virus JalDMZC12/2015(H3N2) son de origen porcino y corresponden al subtipo H3N2 (Cuadro 3).

Cuadro 3. Origen de los segmentos genéticos de influenzavirus porcinos identificados en México.

SEGMENTO	1	2	3	4	5	6	7	8
PROTEÍNA	PB2	PB1	PA	HA	NP	NA	M	NS
A/swine/Mexico/GtoDMZC01/2014(H1N2)	S	S	S	S	S	S	S	S
A/swine/Mexico/GtoDMZC02/2014(H5N2)	C	C	C	C	C	C	C	C
A/swine/Mexico/EdoMexDMZC03/2015(H5N2)	C	C	C	C	C	C	C	C
A/swine/Mexico/GtoDMZC04/2015(H1N1)	H	H	H	H	H	H	H	H
A/swine/Mexico/JalDMZC05/2015(H1N1)	S	S	S	S	S	S	S	S
A/swine/Mexico/GtoDMZC09/2015(H1N1)	S	S	S	S	S	S	S	S
A/swine/Mexico/HgoDMZC11/2015(H3N2)	S	S	S	S	S	S	S	S
A/swine/Mexico/JalDMZC12/2015(H3N2)	S	S	S	S	S	S	S	S

Los aislamientos mexicanos se observan en la columna del lado izquierdo y los segmentos de cada proteína están representadas por un cuadro de color dependiendo el subtipo al que pertenecen: H1N1 (azul), H3N2 (rosa), H1N2 (gris) y H5N2 (naranja), las letras que están dentro de los cuadros de color indican la especie a la que corresponden: swine (S), chicken (C) and human (H).

6.2 Resultados de la prueba de IH

Para determinar la presencia de anticuerpos específicos contra cada virus, se analizaron los sueros de todos los cerdos, confrontándolos con virus homólogos y heterólogos, a través, de la prueba de IH para que ocurra una reacción antígeno anticuerpo y el virus sea neutralizado. Los resultados demostraron que todos los cerdos presentaron títulos de anticuerpos positivos cuando se enfrentaron a sus virus homólogos y negativos cuando se confrontaron a virus heterólogos. (Figura 10).

6.3 Resultados de la variación antigénica

Con los resultados de la IH se calculó el índice de variación antigénica para determinar si existen virus antigénicamente diferentes dentro de la población de estudio. Se considera que dos virus son homólogos si su valor es de 1 y están relacionados antigénicamente si su valor es de 1 ó ≤ 0.5, los valores fuera de éste rango no están relacionados antigénicamente. En éste caso cada suero sólo fue capaz de inhibir la hemoaglutinación del virus homólogo, es decir del virus que se utilizó para la inoculación de cada grupo. En el Cuadro 4 se pueden observar los resultados de la variación antigénica. Con éstos resultados, podemos decir que los cinco virus analizados son antigénicamente diferentes entre ellos.

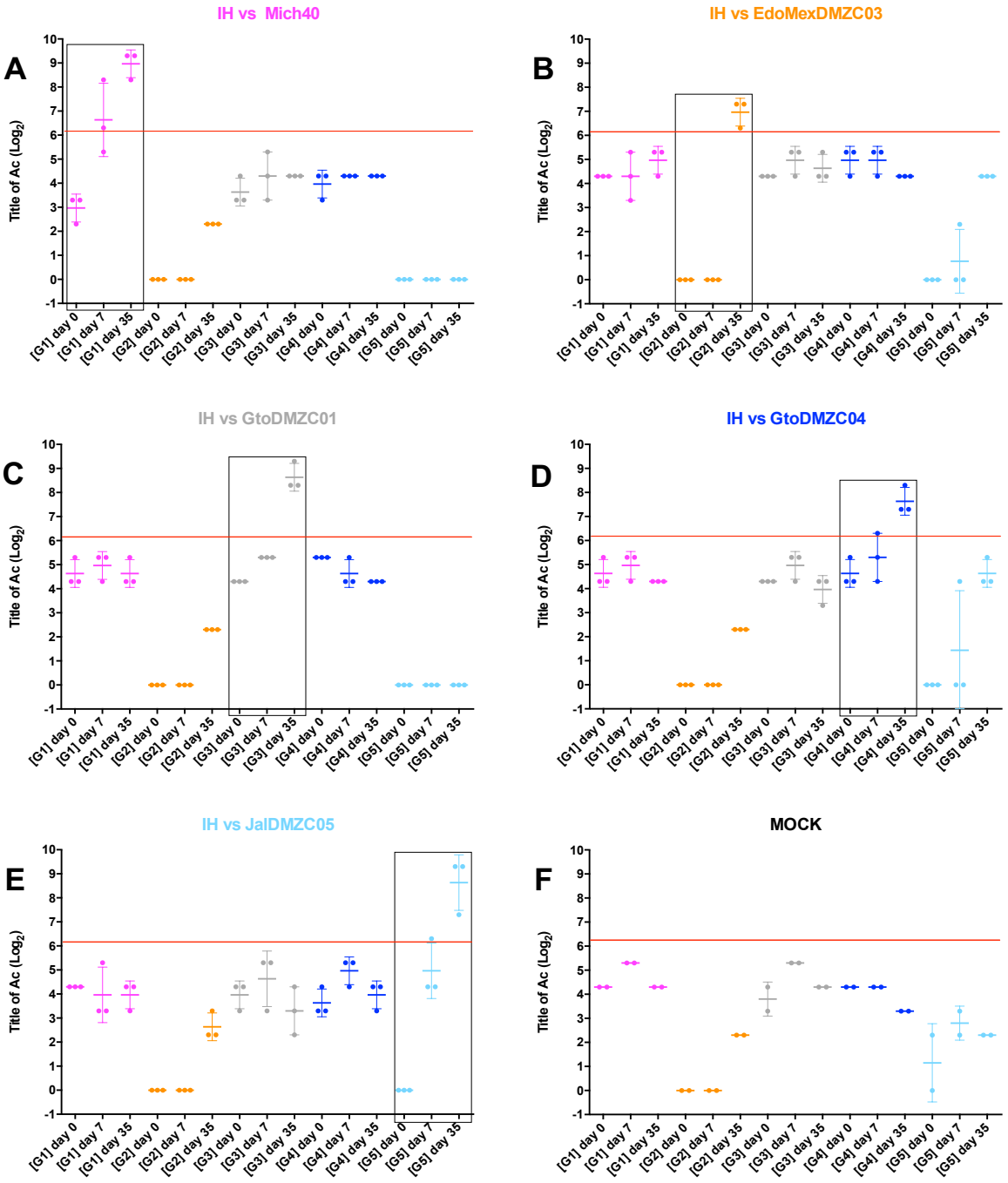

Figura 10. Resultados de la prueba de inhibición de la hemoaglutinación. Los virus empleados fueron inactivados con ß-propiolactona, los cerdos se inocularon vía intramuscular cada 7 días durante 35 días. En las graficas A-F se observan los títulos de Ac (log_2) de los seis grupos analizados [G1-G5 y mock] de los días 0, 7 y 35. La línea roja indica el punto de corte. En el día cero todos los cerdos fueron negativos, en el día 7 los cerdos que tuvieron títulos positivos fueron: dos cerdos del G1 con títulos de 1:80 y 1:320, un cerdo del G4 con título de 1:80 y un cerdo del G5 con título de 1:80. En el día 35 todos los cerdos de los cinco grupos presentaron títulos positivos contra sus virus homólogos con un rango de 1:80 a 1:640, caso contrario a cuando se enfrentaron a sus virus heterólogos en donde todas las respuestas fueron negativas.

Cuadro 4. Resultados del índice de variación antigénica.

	VIRUS														
SUEROS	**Mich40**			**EdoMexDMZC03**			**GtoDMZC01**			**GtoDMZC04**			**JalDMZC05**		
S1 [G1]	**1**	**1**	**1**	0.03	0.04	0.06	0.04	0.04	0.04	0.06	0.04	0.06	0	0	0
S2 [G1]		**1**	**1**	0.04	0.06	0.09	0.06	0.06	0.06	0.09	0.06	0.09	0	0	0
S3 [G1]			**1**	0.03	0.04	0.06	0.04	0.04	0.04	0.06	0.04	0.06	0	0	0
S4 [G2]				**1**	**1**	**1**	0.04	0.04	0.03	0.06	0.04	0.06	0.04	0.03	0.06
S5 [G2]					**1**	**1**	0.06	0.06	0.04	0.06	0.04	0.06	0.04	0.03	0.06
S6 [G2]						**1**	0.06	0.06	0.04	0.09	0.06	0.09	0.06	0.04	0.09
S7 [G3]							**1**	**1**	**1**	0.09	0.04	0.09	0	0	0
S8 [G3]								**1**	**1**	0.09	0.04	0.09	0	0	0
S9 [G3]									**1**	0.06	0.03	0.06	0	0	0
S10 [G4]										**1**	**1**	**1**	0.06	0.06	0.09
S11 [G4]											**1**	**1**	0.04	0.04	0.06
S12 [G4]												**1**	0.09	0.09	0.13
S13 [G5]													**1**	**1**	**1**
S14 [G5]														**1**	**1**
S15 [G5]															**1**

En la columna izquierda se observan los sueros de los grupos G1-G5; los sueros S1-S3 fueron generados con el virus Mich40; los sueros S4-S6 con el virus EdoMexDMZC03; los sueros S7-S9 con el virus GtoDMZC01; los sueros S10-S12 con el virus GtoDMZC04 y finalmente los sueros S13-S15 fueron generados con el virus JalDMZC05. En la fila superior se observan los virus con los que se confronto cada suero. Los valores en negrita representan los resultados homólogos.

7. DISCUSIÓN

En nuestro análisis se identificó que en los virus GtoDMZC01/2014(H1N2), GtoDMZC09/2015(H1N1) y HgoDMZC11/2015(H3N2) existen reordenamientos entre diferentes subtipos. Por otra parte, en los virus GtoDMZC02/2014(H5N2) y EdoMexDMZC03/2015(H5N2) se aprecia el cruce de barrera inter-especie (pasaron íntegros de pollo a cerdo), y el virus GtoDMZC04/2015(H1N1) (paso íntegro de humano a cerdo) y solo dos se encuentran sin cambios el JalDMZC05/2015(H1N1) y el JalDMZC12/2015(H3N2). Diversos análisis filogenéticos sugieren que la transmisión entre especies, ha ocurrido de aves a los cerdos (Karasin, Brown, Carman, & Olsen, 2000; Lee et al., 2009; Peiris et al., 2001), equinos a cerdos (Tu et al., 2009) y de humanos a los cerdos (M. I. Nelson et al., 2014; Martha I. Nelson, Gramer, Vincent, & Holmes, 2012).

Existen trabajos en los que se ha reportado la transmisión de los virus de influenza entre humanos y cerdos, lo que sugiere que estos virus han circulado sin ser detectados. En nuestro trabajo identificamos un subtipo de origen humano en cerdos de México.

Si bien en la población porcina de México se ha reportado que circulan de manera oficial los subtipos H1N1 y H3N2, en el año 2012 se reportó en México por primera vez una seroprevalencia de 80.26% para el subtipo H1N2 (Lara-Puente J., 2012), en otro trabajo se demostró evidencia serológica del subtipo H1N2 desde el año 2010 (Gaitán-Peredo CA, 2016). Más adelante se aísla un virus en la región central de México, la caracterización genética y análisis filogenético determinó que corresponde a un influenzavirus del subtipo H1N2 donde los segmentos eran provenientes de humano y cerdo, además de reportar una seroprevalencia de 26.74% hacia el mismo subtipo (Sánchez-Betancourt, Cervantes-Torres, Saavedra-Montañez, & Segura-Velázquez, 2017). En el presente trabajo se analizó filogenéticamente un influenzavirus H1N2 demostrando que todos sus segmentos corresponden a un origen porcino sin embargo se encuentra reordenado con los subtipos H1N1 y H3N2.

En 2008, en Corea se reportaron dos virus H5N2 que fueron aislados en cerdos. La secuenciación y análisis filogenético de las proteínas determinó que el aislamiento Sw/Korea/C12/08 es un virus totalmente aviar proveniente de aves silvestres (Lee et al., 2009), similar a los virus H5N2 reportados en nuestro estudio en donde todas sus proteínas corresponden a virus aviar, sin embargo en nuestro análisis los subtipos H5N2 se ubicaron en un clado de influenzavirus cuyo origen es de aves de producción (pollo) que fueron aislados y caracterizados en México en los años 1994 y 1995, coincidiendo con brotes de influenzavirus de baja patogenicidad en 1994 y que mutó a H5N2 de alta patogenicidad en 1995 (“Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria,” 2017).

En México existe un evidente escenario de la transmisión de virus de pollo a los cerdos, debido a la existencia de sistemas de producción mixtos en donde dos especies distintas (pollo y cerdo) comparten espacios físicos (INEGI, 2007). Sin embargo, no hay que

descartar la participación de los perros en la diseminación y transmisión interespecies de influenzavirus (Giese et al., 2008; Q. qian Song et al., 2013). En china en 2009, se aisló un influenzavirus H5N2 a partir de un perro que presentaba signos respiratorios (Guang-jian, Zong-shuai, Yan-li, Shi-jin, & Zhi-jing, 2012), más adelante se confirmó la transmisión de este virus de perro a perro (D. Song et al., 2008), posteriormente se observaron manifestaciones de infección en gatos y pollos por este virus H5N2 (Hai-xia et al., 2014).

En el presente estudio fue considerado como positivo un título de igual o mayor a 1:80, lo que aumenta la especificidad de la prueba de inhibición de la hemoaglutinación utilizada (Saavedra-Montañez et al., 2012). Este trabajo muestra que distintos subtipos del virus de influenza porcina que son genéticamente diferentes están circulando en la población porcina de México. Por otra parte, los estudios de inhibición de la hemoaglutinación y de variación antigénica indican una respuesta específica y descartan la reactividad cruzada entre los subtipos.

8. CONCLUSIONES

Con base en el análisis filogenético podemos decir que en la población porcina de México se encuentran circulando influenzavirus que han sufrido reordenamientos de sus proteínas con diferentes subtipos y estos se han adaptado a la población porcina de México. Al mismo tiempo, se ha determinado que algunos influenzavirus humanos y de pollos se han adaptado a los cerdos de México, por ello es importante continuar caracterizando los influenzavirus que circulan en poblaciones humanas y animales que permita identificar las nuevas variantes, particularmente aquellas estrechamente asociados con los humanos para evitar potenciales amenazas zoonóticas. Por otro lado, con los resultados de variación antigénica podremos asegurar que los virus aislados en el presente trabajo son antigénicamente diferentes entre ellos.

9. ARTÍCULO PUBLICADO

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ORIGINAL ARTICLE

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Identification and genomic characterization of influenza viruses with different origin in Mexican pigs

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Abstract

Swine influenza is a worldwide disease, which causes damage to the respiratory system of pigs. The H1N1 and H3N2 subtypes circulate mainly in the swine population of Mexico. There is evidence that new subtypes of influenza virus have evolved genetically and have been rearranged with human viruses and from other species; therefore, the aim of our study was to identify and characterize the genetic changes that have been generated in the different subtypes of the swine influenza virus in Mexican pigs. By sequencing and analyzing phylogenetically the eight segments that form the virus genome, the following subtypes were identified: H1N1, H3N2, H1N2 and H5N2; of which, a H1N1 subtype had a high genetic relationship with the human influenza virus. In addition, a H1N2 subtype related to the porcine H1N2 virus reported in the United States was identified, as well as, two other viruses of avian origin from the H5N2 subtype. Particularly for the H5N2 subtype, this is the first time that its presence has been reported in Mexican pigs. The analysis of these sequences demonstrates that in the swine population of Mexico, circulate viruses that have suffered punctual-specific mutations and rearrangements of their proteins with different subtypes, which have successfully adapted to the Mexican swine population.

KEYWORDS
complete sequences, H5N2, phylogenetic analysis, swine influenza virus Mexico

1 | INTRODUCTION

Swine influenza is a worldwide disease that causes damage to the respiratory system of pigs (Bouvier & Palese, 2008). Influenza viruses belong to the Orthomixoviridae family and have a genome composed of eight segments of ssRNA (–), where each one encodes for one or two proteins (Flint & Racaniello, 2001; King, Adams, Carstens, & Lefkowitz, 2012). The evolution of these viruses occurs through antigenic drift (Drift), characterized by the selection of new strains that contain amino acid changes in hemagglutinin (HA) and neuraminidase (NA) proteins; these changes are responsible for seasonal influenza infections (Carrat & Flahault, 2007; Treanor, 2004). In addition, these viruses can also have genetic rearrangements (Shift) associated with the emergence of pandemic viruses; these occur when a host cell is infected by more than one different virus subtype and the viral genomic segments are rearranged, generating a new combination (Boni, 2008; Webster, Laver, Air, & Schild, 1982; Zambon, 1999). Some accumulated and continuous mutations in the HA protein of the influenza virus have generated new antigenic strains that cause annual epidemics (Liao, Lee, Ko, & Hsiung, 2008). Pigs can naturally or experimentally be infected with viruses of avian or human origin because in the epithelial cells of the trachea, they express avian and

human type receptors (Ito et al., 1998; Scholtissek, Bürger, Bachmann, & Hannoun, 1983), which is why they are considered as mixing containers (Castrucci et al., 1993; Christoph Scholtissek, 1995). Currently, three subtypes of influenza viruses circulate mainly in pigs around the world: H1N1, H3N2 and H1N2 (Chambers, Hinshaw, Kawaoka, Easterday, & Webster, 1991). In the Mexican swine population, it has been reported the circulation of the H1N1 and H3N2 subtypes (Avalos, Sanchez, & Trujillo, 2011; López-Robles, Montalvo-Corral, Caire-Juvera, Ayora-Talavera, & Hernández, 2012); however, there are several studies showing the circulation of new subtypes of influenza virus, which have evolved genetically and have been rearranged with human viruses and from other species (Aguirre et al., 2014; Escalera-Zamudio et al., 2012; Nelson, Culhane et al., 2015; Nelson, Schaefer, Gava, Cantão, & Ciacci-zanella, 2015). On the other hand, there is evidence of the transmission of influenza viruses between different species (bird, pig, equine and human), as well as, rearrangements with different subtypes (H9N2, H4N6, H2N3, H3N8, H3N1, H5N2) in distinct parts of the world (Karasin, Brown, Carman, & Olsen, 2000; Lee et al., 2009; Ma et al., 2007; Peiris et al., 2001; Shin et al., 2006; Tu et al., 2009). These events highlight the requirement for a greater epidemiological surveillance, emphasizing the interest of isolating and characterizing the influenza viruses that circulate currently in the Mexican swine population.

2 | MATERIALS AND METHODS

2.1 | Samples

The research was carried out using 486 pig lung tissue samples from different production units located in twelve states of Mexico (Jalisco, State of Mexico, Guanajuato, Sonora, Michoacan, Puebla, Morelos, Yucatan, Queretaro, Hidalgo, Veracruz and Nuevo Leon) (Figure 1). The criterion for obtaining the samples was to use pigs that presented suggestive signology to infection with influenza virus.

The samples were remitted and processed in the Biosafety Laboratory Level 3 of the Medicine and Zootechnics Swine Department (DMZC) from the Veterinary Medicine and Zootechnics Faculty (FMVZ), National Autonomous University of Mexico (UNAM).

2.2 | RNA extraction

A total of 0.5 gr from each lung tissue was powdered in liquid nitrogen and homogenized in extraction buffer in order to obtain the RNA. RNA extraction was performed with the column technique using the QIAamp Viral RNA mini kit (QIAGEN, Germany) commercial kit, following the manufacturer's specifications. The purified nucleic acid was resuspended in 60 µl of elution buffer and stored at -70°C.

2.3 | qRT-PCR for the M gene

In order to confirm the presence of influenza virus A in the lung samples, a Real Time Reverse Transcription and Polymerase Chain Reaction (qRT-PCR) assay was performed using the FIND-IT INFLUENZA® (Biotecmol, México) commercial kit, directed to the identification of the matrix gene (M) and under the following conditions: 42°C for 30 min for reverse transcription, 95°C for 10 min for initial denaturation, 40 cycles at 95°C for 15 s for denaturation, 60°C for 45 s for annealing and 72°C for 5 min for extension.

2.4 | Sequencing of the Influenzavirus genome

The positive lung samples to the presence of influenza viruses were subjected to RT-PCR for the amplification of the eight gene segments of the virus, using the PathAmp™ FluA Reagents kit (Life Technologies, Carlsbad, CA) and a GeneAmp 9700 end point thermocycler (Applied Biosystems, CA). The RT-PCR products were visualized by electrophoresis in a 0.8% agarose gel.

Once the amplifications were obtained, the eight viral segments were sequenced using an Ion Torrent Genome Machine (PGM) System, following the manufacturer's specifications. Briefly, 100 ng of DNA input was used to prepare a library using the Ion Xpress™ Plus Fragment Library Kit (Life Technologies) by physical fragmentation of genome segments with the Bioruptor® Sonication System, in order to generate fragments of approximately 200 base pairs (bp). DNA fragments were linked to Ion-compatible adapters and amplified using the Ion PGM™Hi-Q™ OT2 Kit (Life Technologies) on an Ion OneTouch™ 2 System. The sequencing reaction was carried out using an Ion 314 Chip v2 and the Ion PGM™ Hi-Q™ Sequencing kit (Life Technologies). The obtained readings were subjected to quality filtering using the FastQC plug-in, and all of them were aligned with a Q-score ≥ 20 and assembled with the AssemblerSPAdes v 5.4.0 program. The average genomic coverage depth of the Ion Torrent PGM was up to 300-fold.

2.5 | Phylogenetic analysis

The obtained sequences were compared with those reported in the GenBank, selecting those with an identity percentage higher than 95%, using BLAST (Basic Local Alignment Search Tool) from the NCBI (National Center for Biotechnology) ("Nucleotide BLAST 2017: Search nucleotide databases using a nucleotide query"). The phylogenetic analysis was performed independently for each genomic segment with the MEGA 7.0.26 software the evolutionary history was inferred using the Maximum Likelihood method based on the General Time Reversible (GTR) model and discrete Gamma distribution with invariant sites was used (Kumar, Stecher, & Tamura, 2016; Nei & Kumar, 2000). The trees were edited with the FIGTREE v1.4.3 program ("FigTree 2017: Molecular evolution, Phylogenetics and Epidemiology").

2.6 | Viral isolation

For viral isolation, sterile 200 µl of lung suspension were inoculated into the allantoic cavity of ALPES1® embryos of 9–11 days, free of specific pathogens (SPF) and incubated at 37°C. The allantoic fluid

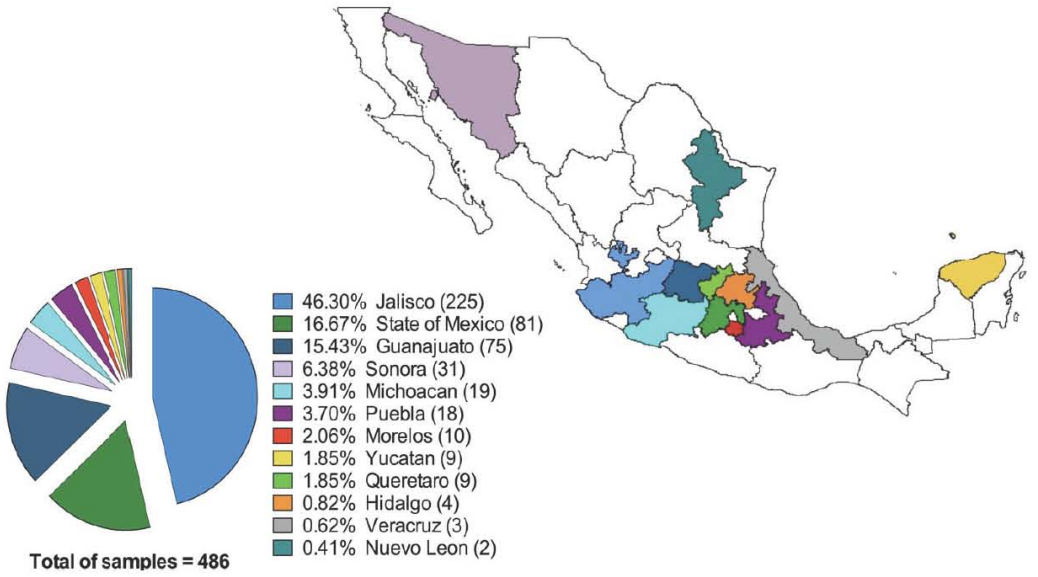

FIGURE 1 Percentage and number of samples per state. Different colors in the map represent the sampled states; whereas the graph shows the percentage and number of samples obtained for each state [Colour figure can be viewed at wileyonlinelibrary.com]

was collected at 24, 48 and 72 hr after inoculation and centrifuged at 423 *g* for 5 min (World Organisation for Animal Health, 2015). The viruses were titrated by hemagglutination (Ramírez, Carreón, & Mercado, 1996).

3 | RESULTS

Of 486 pig pulmonary samples, 10 (0.48%) were positive for influenza virus and 476 (99.52%) negative to the presence of this virus. From the positive samples, the complete genome of 8 influenza viruses was sequenced; three samples corresponded to the H1N1 subtype from the states of Guanajuato (2) and Jalisco (1), and two corresponded to the H3N2 subtype from the states of Jalisco and Hidalgo. An isolated H1N2 sequence was also obtained in the state of Guanajuato, while two H5N2 sequences came from Guanajuato and the State of Mexico (Table 1).

3.1 | Phylogenetic analysis of HA and NA genes

Regarding the evolutionary structure of the inferred topologies of the HA gene, the analysis of the sequences indicated that the HA protein of the GtoDMZC01(H1N2) virus was found in a clade with porcine and human H1N2 influenza viruses, reported in the United States in the period 2005-2010; whereas the HA of the GtoDMZC02(H5N2) and EdoMexDMZC03(H5N2) viruses, were found in a clade of avian influenza H5N2 viruses isolated in Mexico during 1994 and 1995. The HA of the GtoDMZC04(H1N1) virus was found in a clade of H1N1 human influenza viruses, isolated in the United States in 2013 and with one from Mexico isolated in 2014. On the other hand, there is homology between the HAs of JalDMZC05(H1N1) and GtoDMZC09(H1N1) viruses, which were found in a clade of porcine H1N1 influenza viruses isolated in Mexico in the period 2012-2014. The HA of the HgoDMZC11(H3N2) virus was found in a clade of Mexican H3N2 swine influenza viruses isolated in 2012 and 2013. The HA of the JalDMZC12(H3N2) virus was found in a clade with porcine influenza viruses from Canada and the United States, isolated during 2005 and 2006 (Figure 2).

Regarding the evolutionary structure of the inferred topologies from the NA gene, the analysis of the sequences indicated that the NA proteins of the GtoDMZC01(H1N2) and HgoDMZC11(H3N2) viruses were found in a clade with the Mexican H3N2 swine influenza viruses isolated in 2012 and 2013; while the NA of the GtoDMZC02(H5N2) and EdoMexDMZC03(H5N2) viruses were found in a clade of avian influenza H5N2 viruses isolated in Mexico during 1994 and 1995, and with the influenza viruses isolated in the United States in 1994 and 1995. The NA of the GtoDMZC04(H1N1) virus was found in a clade with H1N1 human influenza viruses isolated in the United States during 2013 and 2014. The analysis of the sequences indicated that the NA protein of the JalDMZC05(H1N1) and GtoDMZC09(H1N1) viruses were found in a clade of porcine H1N1 influenza viruses isolated in Mexico in the period of 2012–2014. The NA of the JalDMZC12(H3N2) virus was found in a clade with porcine influenza viruses from Canada and the United States, isolated during 2005 and 2006 (Figure 3).

TABLE 1 Number of positives and negatives according to the origin and subtype of the obtained sequences in our study

	State	(−)*	(+)*	Sequences obtained	Identification in trees**
Slaughterhouse					
	Jalisco	207	1	A/swine/Mexico/JalDMZC12/2015(H3N2)[a] JalDMZC12(H3N2)[b] MH006723-MH006730[c]	JalDMZC12H3N2_swine_MEX_2015.
	State of Mexico	78	0	-	
	Guanajuato	60	1	A/swine/Mexico/GtoDMZC09/2015(H1N1)[a] GtoDMZC09(H1N1)[b] MH006699-MH006706[c]	GtoDMZC09H1N1_swine_MEX_2015.
	Michoacan	3	1	-	
	Hidalgo	2	1	A/swine/Mexico/HgoDMZC11/2015(H3N2)[a] HgoDMZC11(H3N2)[b] MH006715-MH006722[c]	HgoDMZC11H3N2_swine_MEX_2015.
	Puebla	3	0	-	
Farm					
	Sonora	30	1	-	
	Jalisco	16	1	A/swine/Mexico/JalDMZC05/2015(H1N1)[a] JalDMZC05(H1N1)[b] MH013200-MH013207[c]	JalDMZC05H1N1_swine_MEX_2015.
	Michoacan	15	0	-	
	Puebla	15	0	-	
	Guanajuato	11	3	A/swine/Mexico/GtoDMZC04/2015(H1N1)[a] GtoDMZC04(H1N1)[b] MH013192-MH013199[c] A/swine/Mexico/GtoDMZC01/2014(H1N2)[a] GtoDMZC01(H1N2)[b] KT225468-KT225475[c] A/swine/Mexico/GtoDMZC02/2014(H5N2)[a] GtoDMZC02(H5N2)[b] KU141369-KU141376[c]	GtoDMZC04H1N1_swine_MEX_2015. GtoDMZC01H1N2_swine_MEX_2014. GtoDMZC02H5N2_swine_MEX_2014.
	Morelos	10	0	-	
	Queretaro	9	0	-	
	Yucatan	9	0	-	
	Veracruz	3	0	-	
	State of Mexico	2	1	A/swine/Mexico/EdoMexDMZC03/2015(H5N2)[a] EdoMexDMZC03(H5N2)[b] MH013208-MH013215[c]	EMXDMZC03H5N2_swine_MEX_2015.
	Nuevo Leon	2	0	-	
	Hidalgo	1	0	-	
Total		476	10	8	

[a]International classification for each virus. [b]Abbreviations used for each virus. [c]Genbank accession numbers for each virus. [d]by the qRT-PCR assay. [e]Identification used in the construction of inferred phylogenetic trees.

Regarding the origin identification of the eight genetic segments of the influenza viruses isolated in Mexico, it is clear that the eight segments that form the GtoDMZC01(H1N2) virus have a porcine origin; the genes of the PB1, PA, NP and NS proteins correspond to the H1N1 subtype, the NA to the H3N2 subtype and the proteins PB2, HA and M to the H1N2 subtype. In the case of the GtoDMZC02(H5N2) and EdoMexDMZC03(H5N2) viruses, their eight segments have an avian origin and correspond to the H5N2 subtype. On the other hand, the eight segments of the GtoDMZC04(H1N1) virus have a human origin and correspond to the H1N1 subtype; whereas, the eight segments of the JalDMZC05 (H1N1) virus have a porcine origin and also correspond to the H1N1 subtype. The eight segments of the GtoDMZC09(H1N1) virus have a porcine origin and the PB2, PB1, PA, HA, NP, NA and M proteins correspond to the H1N1 subtype; however, NS belongs to the H3N2 subtype. The eight segments of the HgoDMZC11

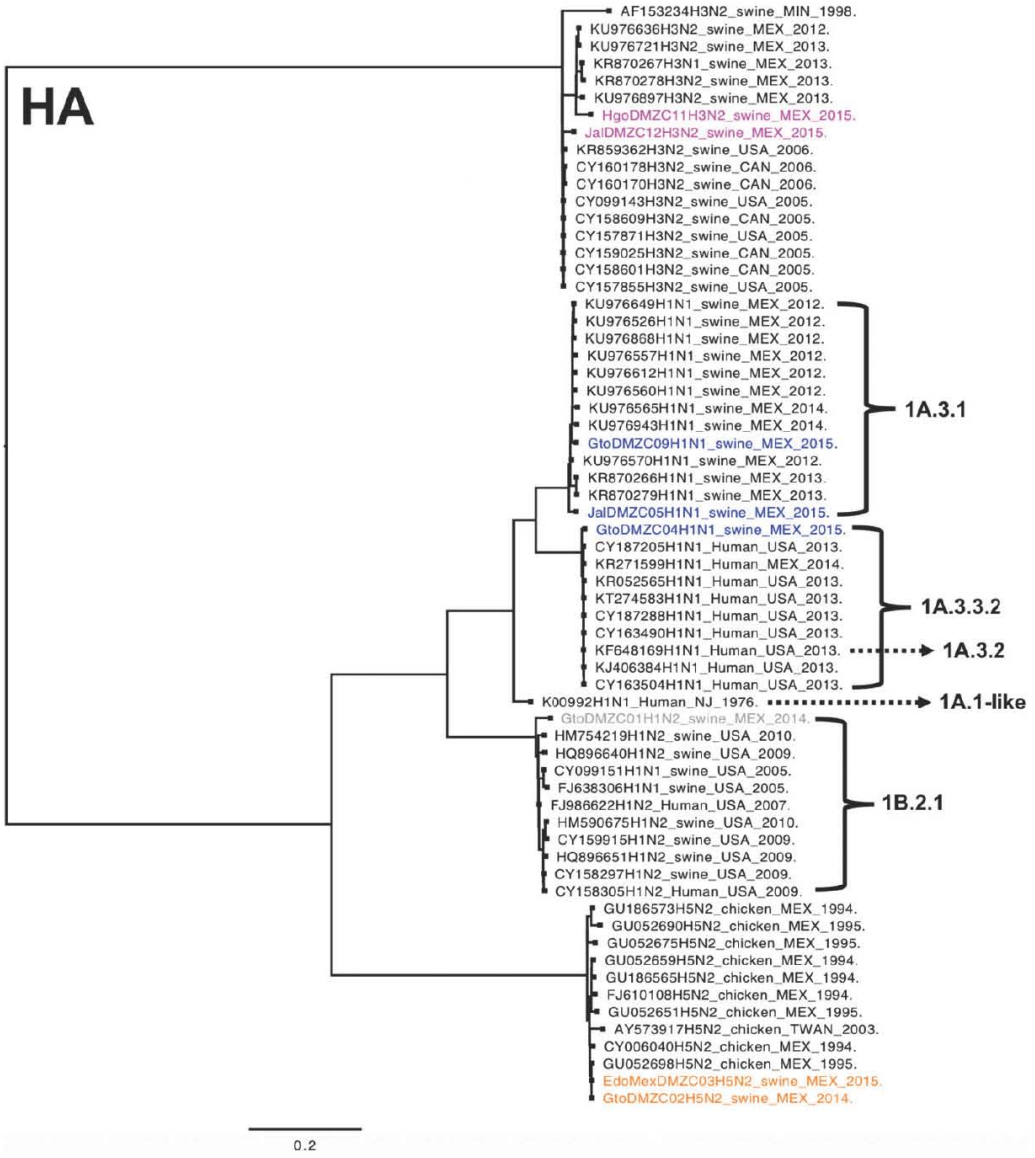

FIGURE 2 The tree is drawn to scale, with branch lengths corresponding to the number of substitutions per site. Mexican isolates are observed in color depending on the subtype: H1N1 (blue), H3N2 (pink), H1N2 (gray), H5N2 (orange). The brackets and arrows indicate the classification of Anderson et al., 2016 [Colour figure can be viewed at wileyonlinelibrary.com]

(H3N2) virus have a porcine origin; the PB1, NP, M and NS proteins correspond to the H1N1 subtype and the PB2, PA, HA and NA proteins to the H3N2 subtype. Finally, the eight segments of the JalDMZC12(H3N2) virus have a porcine origin and correspond to the H3N2 subtype (Table 2).

4 | DISCUSSION

In our study, the central region of Mexico is where the largest number of sequences was obtained; therefore, it could be the region where the largest number of rearrangements occur with viruses of

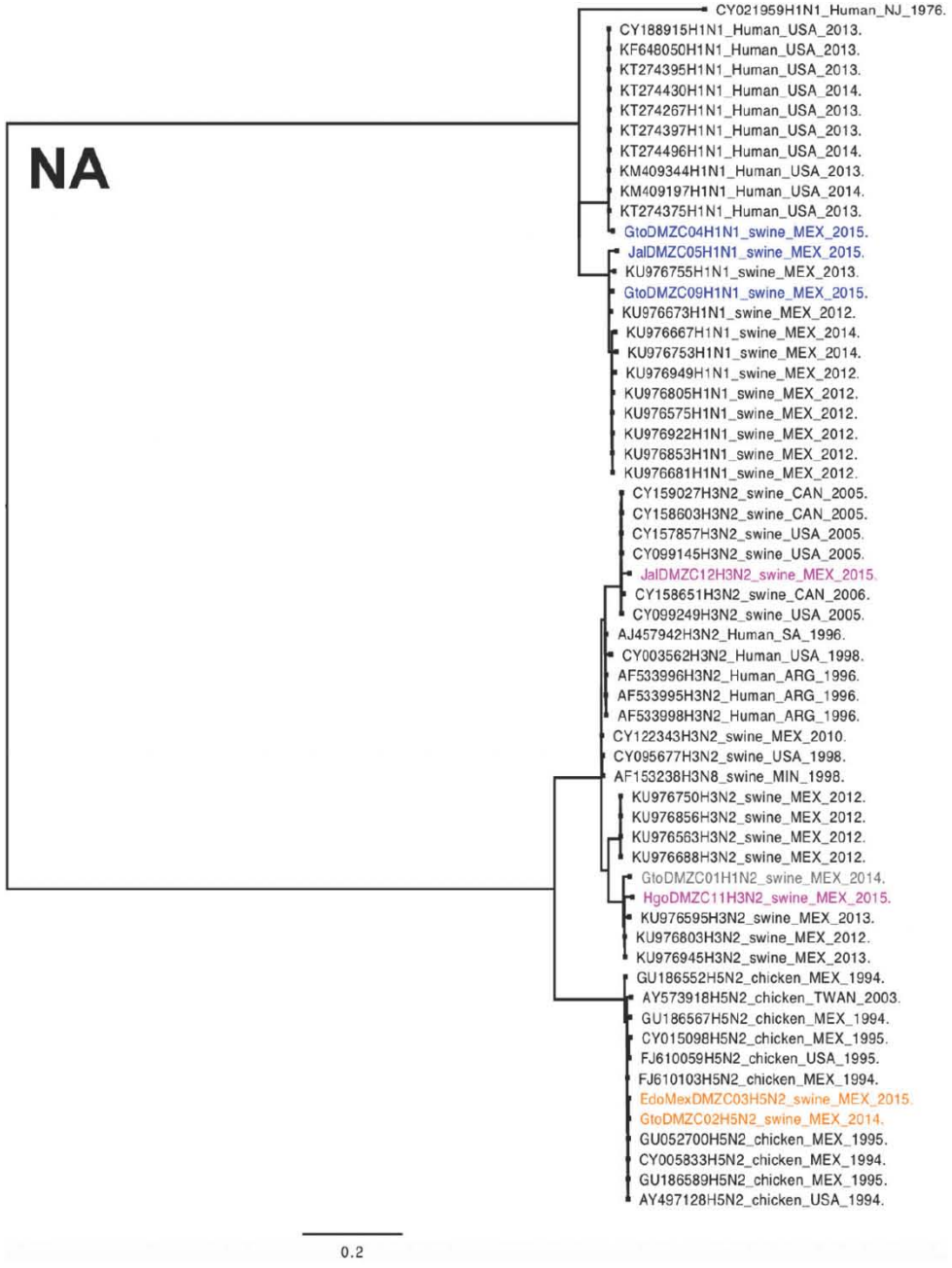

FIGURE 3 The tree is drawn to scale, with branch lengths corresponding to the number of substitutions per site. Mexican isolates are observed in color depending on the subtype: H1N1 (blue), H3N2 (pink), H1N2 (gray), H5N2 (orange) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Origin of the genetic segments of swine influenza viruses identified in Mexico

SEGMENT	1	2	3	4	5	6	7	8
PROTEIN	PB2	PB1	PA	HA	NP	NA	M	NS
A/swine/Mexico/GtoDMZC01/2014(H1N2)	S	S	S	S	S	S	S	S
A/swine/Mexico/GtoDMZC02/2014(H5N2)	C	C	C	C	C	C	C	C
A/swine/Mexico/EdoMexDMZC03/2015(H5N2)	C	C	C	C	C	C	C	C
A/swine/Mexico/GtoDMZC04/2015(H1N1)	H	H	H	H	H	H	H	H
A/swine/Mexico/JalDMZC05/2015(H1N1)	S	S	S	S	S	S	S	S
A/swine/Mexico/GtoDMZC09/2015(H1N1)	S	S	S	S	S	S	S	S
A/swine/Mexico/HgoDMZC11/2015(H3N2)	S	S	S	S	S	S	S	S
A/swine/Mexico/JalDMZC12/2015(H3N2)	S	S	S	S	S	S	S	S

Notes. The Mexican isolates are located in the left side column and the segments of each protein are represented by a color chart depending on the subtype to which they belong: H1N1 (blue), H3N2 (pink), H1N2 (gray) and H5N2 (orange).
The letters that are inside the coloring boxes indicate the species to which they correspond: swine (S), chicken (C) and human (H).

different subtypes, due to production conditions where different species interact in the same habitat (INEGI, 2007).

In our analysis, we identified that in the GtoDMZC01(H1N2), GtoDMZC09(H1N1) and HgoDMZC11(H3N2) viruses, there are rearrangements between different subtypes. On the other hand, the inter-species barrier crossing was appreciated in the following viruses: GtoDMZC02(H5N2) and EdoMexDMZC03(H5N2), which were transferred intact from chicken to pig; and in GtoDMZC04 (H1N1) which was transferred intact from human to pig. Only two viruses were found without change JalDMZC05(H1N1) and JalDMZC12(H3N2). Several phylogenetic analyzes suggest that the transmission between species has occurred from birds to pigs (Karasin et al., 2000; Lee et al., 2009; Peiris et al., 2001), horses to pigs (Tu et al., 2009), and from humans to pigs (Nelson, Gramer, Vincent, & Holmes, 2012; Nelson et al., 2014).

There are several studies that have reported the transmission of influenza viruses between humans and pigs, suggesting that these viruses have circulated without being detected (Cappuccio et al., 2011; Nelson, Culhane et al., 2015; Nelson, Schaefer et al., 2015). In our study, we identified one subtype of viruses of human origin in Mexican pigs and although there are surveillance data regarding influenza viruses in the human population of this country ("Dirección General de Epidemiología, 2017", "FluNet, 2017"). Although, it has been reported that the H1N1 and H3N2 subtypes are officially circulating in the Mexican swine population, until 2012, a seroprevalence of 80.26% was reported for the first time in Mexico for the H1N2 subtype (Lara-Puente, 2012); whereas in other study, the serological evidence for the H1N2 subtype was shown since 2010 (Gaitán-Peredo, 2016). In addition, a virus was isolated in the central region of Mexico, where the genetic characterization and phylogenetic analysis determined that it corresponded to an H1N2 influenza virus, and that the segments came from humans and pigs, besides reporting a 26.74% seroprevalence towards the same subtype (Sánchez-Betancourt, Cervantes-Torres, Saavedra-Montañez, & Segura-Velázquez, 2017). In our study, an H1N2 influenza virus was phylogenetically analyzed, showing that all of its segments have a porcine origin; however, it is rearranged with the H1N1 and H3N2 subtypes.

In 2008, two H5N2 viruses were reported in Korea and were isolated from pigs. The sequencing and phylogenetic analysis of the proteins determined that the Sw/Korea/C12/08 isolate corresponds to a fully avian virus from wild birds (Lee et al., 2009), similar to the H5N2 viruses reported in our study, where all their proteins correspond to an avian virus. However, in our analysis, the H5N2 subtypes were located in a clade of influenza viruses that originated from production birds (chicken) that were isolated and characterized in Mexico during 1994 and 1995. This coincided with several low-pathogenicity outbreaks of influenza viruses in 1994, which mutated into a high pathogenicity H5N2 strain during 1995 ("Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2017").

In Mexico there is an evident scenario for the transmission of chicken virus to pigs, due to the existence of mixed production systems, where two different species (chicken and pigs) share physical spaces (INEGI, 2007). However, the participation of dogs in the dissemination and the interspecies transmission of influenza viruses should not be ruled out (Giese et al., 2008; Song et al., 2013). In China during 2009, an H5N2 influenza virus was isolated from a dog that showed respiratory signs (Guang-jian, Zong-shuai, Yan-li, Shi-jin, & Zhi-jing, 2012). Later on, the transmission of this virus from dog to dog was confirmed (Song et al., 2013). Afterwards, infective manifestations of this H5N2 virus were observed in cats and chickens (Hai-xia et al., 2014).

Our results show that in Mexico, there are viruses with gene rearrangements and viruses that have crossed the inter-species barrier; for this reason, it is important to continue characterizing the A-type influenza viruses circulating in animal populations, in order to identify the new variants, particularly those closely associated with humans to avoid potential zoonotic threats.

Based on the phylogenetic analysis, we conclude that within the Mexican swine population, there are circulating viruses that have undergone punctual mutations and protein rearrangements with different subtypes. On the other hand, we also determined that some segments of human influenza viruses have been rearranged with swine influenza viruses, and these have been detected in Mexican pigs (see figures S1–S6).

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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10. ARTÍCULOS PUBLICADOS DURANTE EL PERIODO DE DOCTORADO

En este trabajo los resultados obtenidos son de gran importancia en el contexto de la transmisión inter-especies debido al papel que desempeñan los cerdos en la distribución del virus y su estrecho contacto que tienen con la población humana y otras especies domésticas. Aunado a éste trabajo se publicaron dos artículos que se realizaron en paralelo a esta investigación.

En el primer trabajo **"*Serological study of influenza viruses in veterinarians working with pigs in Mexico.*"** se evaluó la presencia de anticuerpos contra los subtipos de influenza pH1N1, hH1N1, swH1N1 y swH3N2 en veterinarios especialistas en cerdos en México. Al ser personas que asesoran o atienden granjas porcinas son un grupo que se encuentra en riesgo debido a la exposición ocupacional, la cual incrementa considerablemente el riesgo de infección con el virus de la influenza porcina. Aunque se ha reportado serología positiva y aislamientos esporádicos de virus de la gripe porcina en humanos en varios países, la seroprevalencia de virus de influenza porcina y humana en médicos veterinarios especialistas en cerdos en México sigue siendo desconocida.

En el segundo trabajo **"*Complete genome sequence of a novel influenza H1N2 virus circulating in swine from central bajio region, Mexico.*"** En donde fue posible realizar el aislamiento y secuenciación completa del genoma de un influenzavirus subtipo A H1N2 de un cerdo en Guanajuato e informar su seroprevalencia en 86 municipios en el zona central del Bajío. El análisis filogenético de los ocho segmentos genéticos revelo que es un subtipo H1N2 reordenado, ya que sus genes se derivan de virus de influenza humana (HA, NP, PA) y porcina (M, NA, PB1, PB2 y NS). La seroprevalencia al subtipo H1N2 fue 26.74% en los estados muestreados, siendo Jalisco el estado con mayor seroprevalencia a este subtipo (35,30%). Los resultados aquí reportados. demostrar que este nuevo subtipo de virus de la influenza no registrado anteriormente en México muestra genes internos de otros subtipos virales porcinos aislados en los últimos 5 años, junto con los genes originados por virus humanos, que se distribuyen ampliamente en ésta zona del país.

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ORIGINAL ARTICLE

Serological study of influenza viruses in veterinarians working with swine in Mexico

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Abstract Humans and swine are both affected by influenza viruses, and swine are considered a potential source of new influenza viruses. Transmission of influenza viruses across species is well documented. The aim of this study was to evaluate the seroprevalence of different influenza virus subtypes in veterinarians working for the Mexican swine industry, using a hemagglutination inhibition test. All sera tested were collected in July 2011. The data were analysed using a generalized linear model and a linear model to study the possible association of seroprevalence with the age of the veterinarian, vaccination status, and biosecurity level of the farm where they work. The observed seroprevalence was 12.3%, 76.5%, 46.9%, and 11.1% for the human subtypes of pandemic influenza virus (pH1N1), seasonal human influenza virus (hH1N1), the swine subtypes of classical swine influenza virus (swH1N1), and triple-reassortant swine influenza virus (swH3N2), respectively. Statistical analysis indicated that age was associated with hH1N1 seroprevalence ($P < 0.05$). Similarly, age and vaccination were associated with pH1N1 seroprevalence ($P < 0.05$). On the other hand, none of the studied factors were associated with swH1N1 and swH3N2 seroprevalence. All of the pH1N1-positive sera were from vaccinated veterinarians, whereas all of those not vaccinated tested negative for this subtype. Our findings suggest that, between the onset of the 2009 pandemic and July 2011, the Mexican veterinarians working in the swine industry did not have immunity to the pH1N1 virus; hence, they would have been at risk for infection with this virus if this subtype had been circulating in swine in Mexico prior to 2011.

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Introduction

Influenza A virus (H1N1) was isolated for the first time in swine in 1930 [1]. This virus belongs to the family *Orthomyxoviridae*, which includes two other types, B and C [2–4].

Influenza A viruses infect a large variety of species, including fowl, swine, humans, and horses. Swine play a very important role in interspecies transmission [5]. The interspecies barriers to transmission of influenza virus between humans and swine are not rigorous [6, 7]. Influenza virus infection in swine is relevant, as these animals are capable of expressing the necessary cellular receptors to facilitate recombination of viruses from different species, including humans (N-acetylneuraminic acid-α2,6-galactose) and birds (N-acetylneuraminic acid-α2,3-galactose) [8]. Some avian-type porcine viruses acquire the ability to recognize human receptors, thereby increasing the likelihood of their transmission to humans. As a result, swine are considered the "mixing vessel" for the generation of new reassortant viruses with pandemic potential [9].

In Mexico, records from the period of 2009-2011 made by the General Agency of Epidemiology, through the

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National Epidemiological Surveillance System, indicate that the number of human deaths each year due to infection with non-typed influenza virus was 1744 (1.6%), 410 (0.4%), and 135 (0.1%), respectively [10].

The initial outbreak of the new influenza virus in 2009 had a mortality rate of 0.6% [11], which increased to 2.2% in Mexico [12]. In Mexico, the cases of this new influenza virus, pH1N1, occurring in 2009, were most frequent in the 15- to 50-year-old age group. A possible explanation for this epidemiological distribution could be that adults, particularly those over 60 years of age, have some type of cross-reactive antibody response to the pandemic strains and thus avoided infection [13].

Influenza viruses have been evolving continually. Recent studies have revealed complex relationships among antigenic evolution, genetic evolution, natural selection and the frequent reassortment of influenza viruses [14, 15]. The most common subtypes circulating in humans include H1N1, H1N2, and H3N2, which cause seasonal influenza [16, 17]. It is probable that the influenza viruses circulating in swine, H1N1, H3N2, and H1N2, are closely related to human strains [18]. Subtypes H1N2, H1N1, and H3N2 are the most frequently circulating subtypes in a large number of countries, but neither the human nor the swine H1N2 subtype has been reported in Mexico [19, 20]. Diverse studies have shown that the exposure to swine by personnel working with them serves as a bridge between animals and humans [8, 21]. Although there have been sporadic infections in humans with the classical H1N1 swine and triple-reassortant viruses, only a few cases have been documented in exposed workers [21]. Occupational exposure to swine considerably increases the risk of infection with swine influenza virus subtypes and the pandemic 2009 virus [18, 22]. At a global level, there are antecedents of the transmission of the pandemic virus from humans to swine [22–24]. On the other hand, despite the fact that positive serology and sporadic isolations of the swine influenza virus have been reported for human infections in several countries [16, 25, 26], the seroprevalence in swine-specialist veterinarians in Mexico is not known. The objective of this study was to determine the seroprevalence of human influenza viruses pH1N1 (pandemic) and hH1N1 (seasonal), as well as the swine influenza viruses swH1N1 and swH3N2, in swine-specialist veterinarians in Mexico.

Materials and methods

Study population

We processed a total of 81 serum samples obtained from veterinarians at the Meeting of the Association of Mexican Veterinarians Specialized in Swine (AMVEC in Spanish) held in Puerto Vallarta, Jalisco, Mexico, in July 2011. Of these samples, 79 were obtained from swine specialist veterinarians who came from different federal states of Mexico, whereas the other two were obtained from international attendees (one from the Netherlands and one from Costa Rica). The Executive Council of AMVEC authorized blood sampling. Samples were obtained from veterinarians who provided consent to participate in the study. Samples were collected in Vacutainer® tubes at an approximate volume of 5 mL; the sera were centrifuged and stored at -20°C until hemagglutination inhibition (HI) tests were performed. All participants provided consent for the publication of results; personal data were handled confidentially. Each sampled individual answered a questionnaire, which was then used for the analysis of variables.

Viruses

The following influenza subtypes were used as antigens: seasonal human influenza (hH1N1) A/Mexico/INER1/2000 (H1N1) (GenBank accession number JN086908), pandemic influenza (pH1N1) A/Mexico/LaGloria-3/2009 (H1N1) (GenBank accession number CY077595), classical swine (swH1N1) A/swine/New Jersey/11/76 (H1N1) (GenBank accession number K00992), and a triple-reassortant swine influenza virus (swH3N2) A/swine/Minnesota/9088-2/98 (H3N2) (GenBank accession number AF153234). Viruses were grown according to a previously described protocol [27].

Hemagglutination inhibition assay

We used the procedure established by the World Health Organization [28], with the following modifications: hemagglutinating units (HAU) were adjusted to 8. Sera were inactivated at 56 °C and adsorbed with kaolin and 5% chicken erythrocytes. Briefly, the serum was diluted using twofold serial dilutions from 1:20 to 1:5120. Afterwards, the diluted sera were mixed with 8 HAU of each virus and incubated for 30 min at ambient temperature (21 °C). At the end of this incubation, 0.5% chicken erythrocytes were added, and the mixture was left to incubate for an additional 30 min at ambient temperature. Readings were taken at the end of the incubation period. Titers of sera were considered positive if they were $\geq$1:80.

Statistical analysis

Antibody titers were normalized using the $\log_2$ transformation. A generalized linear model was used to assess seropositivity and its association with different factors. During the survey, we collected the following data: sex, age, number of years of veterinary practice with swine,

vaccination history against seasonal human influenza and/or pandemic influenza virus, units of porcine production managed, production region in Mexico (northwestern, northeastern, central-western, central, and southeastern), and the level of biosecurity on the farms (low, medium, or high). Only the following variables were statistically analysed: sex, vaccination, and level of biosecurity (because only these variables had complete data). The missing variables were included only in the descriptive analysis. All statistical analyses were performed using JMP® 9.0 software (SAS Institute Inc., Cary, NC, USA).

Results

The number of seropositive subjects, the average titer, the seroprevalence, and the percentage seropositive for pH1N1, hH1N1, swH1N1, and swH3N2 are presented in Table 1. Among the participating veterinarians, 79% were men, with an average age of 42.2 years (range 20-63 years), 21% were women, and their average age was 35.8 years (range 23-53 years). Among the final sample of 81 surveyed veterinarians, 79 were Mexicans and two were foreigners; their average age was 40.8 years, and the average time of exposure was 15.2 years. These data are shown in Table 1.

Statistical analysis revealed that in the entire group of veterinarians (vaccinated and unvaccinated, n = 81), the age and vaccination effects had a significant association with pH1N1 seropositivity ($P < 0.049$, and $P < 0.0001$, respectively). These data indicate that a higher age of the interviewed veterinarians was associated with a larger number of samples with antibodies and that the vaccinated individuals tested positive. Age was significantly associated with hH1N1 seropositivity ($P < 0.0002$), and this finding indicates that seropositivity for human influenza viruses increased with age; this observation is independent of whether people work with swine, as these viral subtypes circulate in the human population. Regarding swH1N1, only a marginal effect was found ($P = 0.057$) to be associated with seroprevalence, suggesting that older veterinarians have had more contact with swine over time and therefore have a higher probability of a humoral immune response against swH1N1. For subtype swH3N2, none of the assessed variables were significantly correlated with seropositivity ($P > 0.05$). The result was not significant, as this subtype is less common in swine [29]; thus, there is a lower probability of transmission to humans.

Among vaccinated veterinarians (vaccinated individuals, n = 29), for subtype pH1N1, age had a significant association ($P = 0.048$). In Mexico, there is a vaccination campaign that considers the elderly as candidates for vaccination; hence, the data are consistent with the observation that there is a higher seropositivity for the pandemic virus in older veterinarians. For subtype hH1N1, the biosecurity level was significant ($P = 0.0094$). Although this variable was statistically significant, it does not correspond to an expected biological effect. For subtype swH1N1, age was significant ($P = 0.012$), and the age effect corresponds to that described previously. For subtype swH3N2, none of the variables were significant ($P > 0.05$) in the studied group.

When analysing only unvaccinated veterinarians (unvaccinated, n = 52), age was statistically significant for subtype hH1N1 ($P = 0.0006$); in this group, the level of significance is consistent with expected behaviour, as exposure to the seasonal virus is greater with time, whereas for subtypes swH1N1 and swH3N2, none of the variables were statistically significant ($P > 0.05$). The *P*-values for the different effects among the viruses are shown in Table 2.

Positive sera from swine analysed for subtypes hH1N1 and swH1N1 yielded higher titers (1:1280) as compared to swH3N2 and pH1N1 (1:160 and 1:320 respectively). The frequency and average of the antibody titers for each subtype are shown in Table 3. On the other hand, Tukey test results indicated that the titer of the antibodies for subtype hH1N1 did not differ significantly from those observed for subtype pH1N1. However, the titer recorded for hH1N1 differed significantly from those for swH1N1 and swH3N2 ($P < 0.05$). No significant differences were observed among the values for pH1N1, swH1N1, and swH3N2 ($P > 0.05$) (Table 3).

Table 4 summarizes the results of tests of the antibody response when two, three, or four subtypes were present simultaneously. For pH1N1, when in combination with subtype hH1N1, there were 10 veterinarians with a positive humoral response to these two subtypes in which the antibody titer against the hH1N1 virus was five times higher than that against pH1N1.

In seven of the veterinarians in whom antibodies were found against both pH1N1 and swH1N1 simultaneously, the swH1N1 virus generated a response with titers two times higher than those of pH1N1. In combination with swH3N2, there were three veterinarians who were positive for this combination of subtypes, and in the three cases, the titer was higher for subtype pH1N1.

In individuals with antibodies against both hH1N1 and swH1N1, there were 33 veterinarians with a positive response; among these veterinarians, 16 had higher titers for subtype hH1N1. When swH3N2 simultaneously was present, there were eight veterinarians with a positive response; among these veterinarians, seven had higher titers for subtype hH1N1.

In individuals with antibodies against both swH1N and swH3N2, there were six veterinarians with a positive

Table 1 Number seropositive, average titer, seroprevalence and percentage of veterinarians according to variable and virus subtype

Variable	*n*	pH1N1	hH1N1	swH1N1	swH3N2
Total	81				
Seropositive		10	62	38	9
Seroprevalence % [95% CI]		12.3 [6.5-20.3]	76.5 [62.6-81.2]	46.9 [34.5-55.2]	11.11 [5.6-18.9]
Average titer		1:168	1:260	1:189	1:98
Biosecurity level at the farm					
Low	5	0 (0)	3 (60)	2 (40)	0 (0)
Medium	15	1 (6.7)	11 (73.3)	6 (40)	3 (20)
High	61	9 (14.7)	48 (78.7)	30 (49.2)	6 (9.8)
Influenza vaccination					
Yes	29	10 (34.5)	22 (75.9)	15 (51.7)	4 (13.8)
No	52	0 (0)	40 (76.9)	23 (44.2)	5 (9.6)
Sex**					
Men	64 (79)	9 (11.1)	52(64.2)	31 (38.3)	9 (11.1)
Women	17 (21)	1 (1.2)	10 (12.3)	7 (8.6)	0 (0)
Mean age	40.8	47.6	43.3	43.1	45.1
Age (ranges)**					
20-30 years		0	7	5	2
31-41 years		2	18	9	2
42-52 years		6	28	18	4
53-63 years		2	9	6	1
Mean exposed years**	15.2	23.6	17.1	18.1	16.7
Region**					
Northwestern	8 (9.9)	2 (2.5)	6 (7.4)	4 (4.9)	1 (1.2)
Northeastern	1 (1.2)	0 (0)	1 (1.2)	1 (1.2)	0 (0)
Central-western	37 (45.7)	5 (6.2)	31 (38.3)	18 (22.2)	3 (3.7)
Central	28 (34.5)	1 (1.2)	18 (22.2)	12 (14.8)	4 (4.9)
Southeast	3 (3.7)	0 (0)	3 (3.7)	1 (1.2)	0 (0)
International	2 (2.5)	1 (1.2)	2 (2.5)	1 (1.2)	1 (1.2)
Units of porcine production managed**					
0	9	0	6	3	0
1	23	2	13	7	1
2	23	2	11	7	3
3	27	4	12	8	3
4	10	0	5	4	1
5	17	1	8	7	1
>6	9	1	6	2	0

Missing data are due to failure to fully answer the questionnaire. ** these variables were not included in the statistical analysis
[ ] = confidence interval. () = percentage of positive veterinarians to each variable

response; among these veterinarians, four had higher titers of subtype swH1N1.

At no time were the titers to subtype swH3N2 higher than those to the other subtypes.

In the case where veterinarians generated antibodies simultaneously against three subtypes, seven were positive against pH1N1, hH1N1, and swH1N1; in this case, two veterinarians had higher titers to subtype swH1N1. On the other hand, three veterinarians had antibodies against pH1N1, hH1N1, and swH3N2; in this case, subtype hH1N1 was the one with the highest titer compared to the other two. In addition, three veterinarians had antibodies against pH1N1, swH1N1, and swH3N2; in this case, the titers against swH1N1 were the highest. Three veterinarians had antibodies against hH1N1, swH1N1, and swH3N2; here, the titers against subtype hH1N1 were the highest.

Table 2 Level of significance of the studied effects according to virus subtypes analyzed in the different groups of veterinarians

Variable	pH1N1	hH1N1	swH1N1	swH3N2
All veterinarians (n = 81)				
Age	[0.0491]*	[0.0002]*	[0.0569]°	[0.3386]
Biosecurity level at the farm	[0.2742]	[0.1977]	[0.6749]	[0.2705]
Vaccinated	[<0.0001]*	[0.4215]	[0.7853]	[0.5959]
Vaccinated veterinarians (n = 29)				
Age	[0.0476]*	[0.3229]	[0.0122]*	[0.1716]
Biosecurity level at the farm	[0.4486]	[0.0094]**	[0.1071]	[0.6056]
Unvaccinated veterinarians (n= 52)				
Age	[NC]	[0.0006]*	[0.6357]	[0.8748]
Biosecurity level at the farm	[NC]	[0.6338]	[0.4272]	[0.4945]

[P value], *, Statistically significant; ° = Marginal; NC = noncomputable; **, this effect although registered as significant, does not have epidemiological relevance, as it is not a dependent variable

Table 3 Average and frequency of antibody titers against the four influenza subtypes in the sampled population

Titer	pH1N1	hH1N1	swH1N1	swH3N2
<40	62	5	33	57
1:40	9	14	10	15
1:80	3	16	18	7
1:160	5	22	14	2
1:320	2	11	3	-
1:640	-	12	2	-
1:1280	-	1	1	-
Average*	$(7.1)^{a,b}$	$(7.6)^{a}$	$(7.0)^{b}$	$(6.4)^{b}$

* Average based on log2 transformed data

Different superscript letters indicate statistically significant difference ($P < 0.05$)

Finally, three veterinarians had antibodies against pH1N1, hH1N1, swH1N1, and swH3N2 simultaneously; in this case, the subtype swH1N1 generated the highest titer.

Discussion

Serological evidence of transmission of swine influenza virus subtypes among swine specialist veterinarians is considered positive when the titer is at least 1:40 [30]. The possibility of cross-reactivity among hH1N1, pH1N1 and, swH1N1 exists. However, starting with a dilution of 1:160, hH1N1 becomes the most frequently observed virus. It has also been observed that the same sample can be positive for all three subtypes; however, the antibody titers against one subtype were much higher than those against the other subtypes, and similar titers for the three subtypes were never observed. A person can have antibodies against one or more subtypes at the same time, and the existence of cross-reactivity with different subtypes is also possible; however, the higher titers indicate a specific response and appear to rule out cross-reactivity among the subtypes. Instead, the presence of antibodies against more than one subtype may be a result of a recent infection [22]. In the present study, a titer of $\geq$ 1:80 was considered the cutoff point, thereby increasing the specificity of the HI test used [27].

Influenza infection is a global endemic disease [31]. However, there are no previous reports of serological studies on veterinarians in Mexico. In the present serological evaluation, we used only lineages that circulate in Mexico. Other studies in Mexico have used strains of European and North American lineages, such as A/Bayern/7/95 (H1N1) (GenBank: EF566037.1), A/Sydney/5/97 (H3N2) (GenBank: EF566075.1), A/Swine/Wisconsin/238/97 (H1N1) (GenBank: AF222033.2), A/Swine/Minnesota/593/99 (H3N2) (GenBank: AF251427.2), A/NewCaledonia/20/99 (H1N1) (GenBank: AY289929.1), A/Panama/2007/99 (H3N2) (GenBank: DQ487340), and A/Swine/England/163266/87 (H3N2) (GenBank: CY115996) [32, 33]; therefore, a higher seropositivity was observed in the present study.

In studies by López Robles et al., Ayora-Talabera et al., and Fragaszy et al [22, 32, 33], the average seroprevalence for subtype swH1N1 was lower than that for swH3N2, and these researchers concluded that this subtype circulates more frequently than swH1N1. This conclusion is in contrast to our assessment that antibodies against subtype swH1N1 were present in veterinarians at higher frequency and with higher titers than those against swH3N2, similar to what has been observed in swine on farms [29].

In Iowa, a seroprevalence of 12.4% against swH1N1 has been observed in individuals exposed to swine, and this was reportedly associated with variables such as sex, age, years of porcine production management, number of days working with swine, use of protection equipment, recent

Table 4 Combination of subtypes that simultaneously generated a humoral response in the veterinarians, the number of times each subtype was associated with the highest titer, and the average titer of antibodies

Subtypes that simultaneously generated positive results	Times each subtype was associated with the highest titer (average)*	Total average antibody titer (log_2)			
		pH1N1	hH1N1	swH1N1	swH3N2
Two subtypes					
pH1N1-hH1N1 (10)	2 (7.82) - 5 (8.92)	7.22	7.82	NA	NA
pH1N1- swH1N1 (7)	2 (7.82) - 2 (9.82)	7.46	NA	7.75	NA
pH1N1-swH3N2 (3)	3 (7.98) - 0	7.98	NA	NA	6.65
hH1N1-swH1N1 (33)	16 (8.57) – 8 (8.45)	NA	7.84	7.2	NA
hH1N1-swH3N2 (8)	7 (8.46) - 0	NA	8.19	NA	6.44
swH1N1-swH3N2 (6)	4 (8.32) - 0	NA	NA	7.82	6.65
Three subtypes					
pH1N1-hH1N1-swH1N1 (7)	1 (8.32) – 3 (8.98) – 2 (9.82)	7.46	8.32	7.75	NA
pH1N1-hH1N1-swH3N2 (3)	1 (8.32) – 1 (9.32) – 0	8.65	7.98	NA	6.65
pH1N1-swH1N1-swH3N2 (3)	1 (8.32) – 1 (10.32) – 0	7.98	NA	8.3	6.65
hH1N1-swH1N1-swH3N2 (3)	1 (9.32) – 1 (8.32) – 0	NA	7.65	7.32	6.32
Four subtypes					
pH1N1-hH1N1-swH1N1-swH3N2 (3)	1 (8.32) – 1 (9.32) – 1 (10.32) - 0	7.98	8.65	8.32	6.65

*Average is based on log2 transformed data, and the missing data correspond to equal titers. NA = not applicable

exposure, number of swine on the farm, and type of farm [34]. In Wisconsin, different variables were analysed, and age (≤50 years) and vaccination history were significantly associated factors, whereas the number of working hours was not [30]. In these previous studies, a titer of 1:40 was used as the cutoff, in contrast to the 1:80 used in the present study. In July 2009, a study was conducted on a farm in Alberta, Canada, and it was determined that workers exposed to swine had a seroprevalence of 67% against subtype pH1N1 (including permanent staff, researchers, and students) [35]. This finding is different from our results of seroprevalence in veterinarians (12.3%), indicating that veterinarians may be less susceptible than personnel exposed to swine on a daily basis. In veterinarians, a seroprevalence of 22.7% against subtype swH3N2 has been reported in Germany for the period from December 2007 to April 2009 [16].

We found seropositivity against the four analysed subtypes. The average antibody titer was highest for subtype hH1N1, followed by subtype swH1N1. Therefore, our results imply that seropositivity for hH1N1 is achieved by transmission of the infection among humans, whereas seropositivity for pH1N1 virus originated from vaccination, and seropositivity for swH1N1 was due to exposure to swine [16, 21, 32].

In our study, we observed lower seroprevalence (11.1%) for the swH3N2 virus. However, since seropositivity was observed, the possibility of transmission of the swine virus to humans cannot be ignored. We detected antibodies against swH3N2 in veterinarians who work with swine; however, there is the possibility of cross-reactivity with viruses H3N2 that circulate in humans, and there are studies supporting this notion [36].

Limitations of the present study include the use of old strains and the omission of a control group of non-veterinarians. In addition, we obtained more male samples than female samples because there are more men than women involved in porcine production in Mexico. The largest number of seropositive samples was for hH1N1, indicating that this subtype is circulating among the population of veterinarians; however, the pH1N1-positive results corresponded to veterinarians who had been vaccinated against this subtype. We observed that most of the veterinarians who answered the questionnaire work at farms with a high biosecurity level. In addition, the largest number of positive samples corresponded to subtype hH1N1. Regarding biosecurity, there were no significant effects; however, numerically, there was a higher proportion in high-biosecurity farms. According to our classification, farms with a higher level of biosecurity correspond to farms with a larger number of animals. The high density of swine favors dissemination of infections. Although it is true that with higher biosecurity (an arbitrary and not objective parameter) the entry of pathogens is diminished, it does not mean that pathogens already inside the farm are reduced, especially in farms where many infections are endemic, as is the case of swine influenza virus.

The number of positive results for influenza virus obtained in this study was higher in the central and central-western regions of the country; these two regions comprise more than 70% of the porcine production of the country [37].

One of the recommendations given at the time of the pandemic was that persons with flu-like symptoms should stay home from work. In Mexico, it was recommended that workers in contact with swine receive the vaccination against human influenza each year. In Mexico, the populations that are usually vaccinated are children and the elderly. According to the results obtained in the present study, the seroprevalence against the pH1N1 virus in the swine-specialist veterinarians (12.3%) was lower than that in the general population (20%) [38]. The results of this study are consistent with those of other studies in which there is evidence of dissemination of the virus (swine influenza H1N1 and swine influenza H3N2) from swine to veterinarians [18, 39].

Age and vaccination were significantly associated with pH1N1 seropositivity. Similarly, age was significantly associated with hH1N1 seropositivity and swH1N1 seropositivity. None of the variables were significantly associated with swH3N2 seropositivity, as sera positive for swH3N2 were scarce. These findings fail to establish a relationship among age, vaccination, biosecurity level, and sex. Despite having worked directly with swine for years, the veterinarians' level of infection for the latter subtype was low. Even though seropositivity was not significant, veterinarians could still be infected with this subtype. The lack of a significant effect of subtype swH3N2 suggests that there was no cross-reactivity antigenicity exists in the veterinarians vaccinated with the vaccinal component H3N2, which is usually included in the vaccine applied to humans.

Our results indicate that among veterinarians working with swine in Mexico, the risk of infection with influenza viruses pH1N1, hH1N1, and swH1N1 is higher in older veterinarians; however, the pH1N1 and hH1N1 seropositivity values are not known for those working on swine farms.

The present findings could prove instrumental in determining the seroprevalence of swine influenza virus among veterinarians in Mexico and in establishing associated risk factors. The pH1N1-positive results from veterinarians are due to vaccinations, whereas unvaccinated veterinarians tested negative for this subtype. Hence, it can be concluded that after the 2009 influenza pandemic, the veterinarians working in porcine production in Mexico have not been at risk for possible transmission of the pandemic virus to swine, at least, not at the moment. Studies by Nelson and coworkers have clearly indicated the transmission of the influenza virus from humans to swine [14, 23, 24]; however, in our work, the detection of antibodies to the pH1N1 subtype indicates that these originated from vaccination, at least in this group of veterinarians.

If veterinarians were transmitting the pandemic virus, the number of positive samples to this subtype would be similar to or higher than that of seasonal hH1N1 influenza, but this phenomenon did not occur. This hypothesis was proposed after the 2009 pandemic and may be rejected according to the present results.

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Compliance with ethical standards

This article does not contain any studies with animals performed by any of the authors.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Universidad Nacional Autónoma de México and the Consejo Nacional de Ciencia y Tecnología (Mexico).

Informed consent Informed consent was obtained from all individual participants included in the study.

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ORIGINAL ARTICLE

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Complete genome sequence of a novel influenza A H1N2 virus circulating in swine from Central Bajio region, Mexico

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Summary

The aim of this study was to perform the complete genome sequence of a swine influenza A H1N2 virus strain isolated from a pig in Guanajuato, México (A/swine/Mexico/GtoDMZC01/2014) and to report its seroprevalence in 86 counties at the Central Bajio zone. To understand the evolutionary dynamics of the isolate, we undertook a phylogenetic analysis of the eight gene segments. These data revealed that the isolated virus is a reassortant H1N2 subtype, as its genes are derived from human (HA, NP, PA) and swine (M, NA, PB1, PB2 and NS) influenza viruses. Pig serum samples were analysed by the hemagglutination inhibition test, using wild H1N2 and H3N2 strains (A/swine/México/Mex51/2010 [H3N2]) as antigen sources. Positive samples to the H1N2 subtype were processed using the field-isolated H1N1 subtype (A/swine/México/Ver37/2010 [H1N1]). Seroprevalence to the H1N2 subtype was 26.74% in the sampled counties, being Jalisco the state with highest seroprevalence to this subtype (35.30%). The results herein reported demonstrate that this new, previously unregistered influenza virus subtype in México that shows internal genes from other swine viral subtypes isolated in the past 5 years, along with human virus-originated genes, is widely distributed in this area of the country.

KEYWORDS
genome, H1N2, seroprevalence, swine influenza

1 | INTRODUCTION

The influenza virus is a member of the *Orthomyxovirus* family with a segmented RNA genome. Type A swine influenza viruses (swIAV) are further subclassified according to the hemagglutinin and neuraminidase proteins they express. Pigs are naturally susceptible to infection by human and avian H1N1, reassortant (r) H3N2 and rH1N2 subtypes (Kitikoon et al., 2012). Swine influenza viruses are a cause of public health concern, and pigs play a unique role in the global epidemiology of human influenza. Swine respiratory tract epithelial cells have receptors for both avian and mammalian influenza viruses (Ito, Kawaoka, Nomura, & Otsuki, 1999); therefore, pigs can potentially serve as mixing vessels for the development of new reassortant virus strains with a pandemic potential in the human population. On several occasions, avian influenza viruses (AIVs) of various subtypes (H1N1, H3N2, H3N3, H4N6, H5N2, H9N2) have been isolated from pigs on the field (De Vleeschauwer et al., 2009). An example of this occurred in China in 2012: two triple-reassortant H1N2 influenza viruses were isolated from swine samples, with an HA gene derived from an Eurasian avian-like swine H1N1 strain, an NA gene from a North American swine H1N2, and the six internal genes from the pandemic 2009/H1N1 viruses (Qiao et al., 2014). In addition, pig susceptibility to AIVs has been confirmed experimentally. Intranasal inoculation to pigs with most AIVs, both with low and high pathogenicity, generally resulted in moderate nasal swab virus titres and seroconversion (Hinshaw, Webster, Easterday, & Bean, 1981; Lipatov et al., 2008).

Herein, we report the genetic characterization of an A H1N2 virus isolated from a pig in a farm at Central México. Phylogenetic analysis of the obtained sequences revealed that all virus segments are from swine and human origin. Additionally, 26.74% of serum samples from farms located in this region, where this H1N2 virus was first isolated, showed specific antibodies against it, indicating the magnitude of the circulation of this virus in the region.

2 | MATERIALS AND METHODS

2.1 | Sample collection, viral isolation and RNA extraction

Lung tissue samples were collected from killed pigs at a slaughterhouse in Guanajuato, México, on November 2014. Samples were kept under refrigeration and processed for RT-PCR and viral isolation in a biosafety level 3 (BSL3) laboratory at Departamento de Medicina y Zootecnia de Cerdos (DMZC), Facultad de Medicina Veterinaria y Zootecnia (FMVZ), Universidad Nacional Autónoma de México (UNAM). Briefly, lung tissue samples were homogenized by grinding in liquid nitrogen, and the triturated suspension was centrifuged at 1,500 *g* for 10 min. Supernatants were used for RNA extraction using the QIAamp viral RNA mini kit (Qiagen, Düsseldorf, Germany) and for virus isolation. To confirm the presence of swIAV in lung samples, a real-time PCR assay designed by Biogénica® was performed to identify the viral gene M. Sterile 200-µl aliquots of lung suspension were inoculated into the allantoic cavity of specific pathogen free (SPF), 9–11 days ALPES1® chicken embryos and incubated at 37°C. The fluid was collected at 24, 48 and 72 hr after inoculation and centrifuged at 1645 *g* for 5 min. Viruses were titrated by hemagglutination.

2.2 | Sequencing

Viral RNA was extracted directly from the isolate after three passages using the Qlamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. All eight influenza gene segments were amplified with PathAmp™ FluA Reagents (Life Technologies, Carlsbad, CA), a two-step multiplex reverse-transcription polymerase chain reaction (PCR) rendering a six-band profile on a standard 1.5% agarose gel. The quality, quantity and integrity of total DNA were evaluated using the High Sensitivity DNA Kit (Agilent, Santa Clara, CA). Then, a library was prepared with Ion Xpress™ Plus Fragment Library Kit (Life Technologies) by physical fragmentation of genome segments with the Bioruptor® Sonication System, to generate fragments of approximately 200 base pairs (bp); DNA fragments were immediately linked to Ion-compatible adapters and nick-repaired to complete the linkage between adapters and the DNA inserts forming the library. A template was performed by emulsion PCR with an automated template preparation using the Ion OneTouch™ 2 system (Life Technologies). Finally, DNA-positive beads were recovered, enriched and subjected to sequencing with the Ion PGM™ sequencer using an Ion 314 Chip. The obtained reads were subjected to quality filtering using the FastQC plug-in v 0.10.1, available from ioncommunity.lifetechnologies.com. All reads with a Q-score ≥ 20 (399,344) were aligned with 17 complete swIAV sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nphselect.cgi?go=genomeset) from 2009 to date. The sequencing protocol generated an average coverage for each genomic segment ranged from 806 to 7,116 reads. The genomic average depth of coverage of the Ion Torrent PGM was up to 305-fold.

2.3 | Phylogenetic analysis

To explore the evolutionary origins of the H1N2 isolate, a tree for each genomic segment was inferred based on nucleotide sequences from GenBank that had sequence identity higher than 95% using BLASTn, published by NCBI (National Center for Biotechnology Information). The HA phylogenetic tree included 33 sequences from GenBank, and 23 sequences of the previously described H1 clusters: H1α, H1β, H1γ, H1δ1, H1δ2, H1pdm09 (Grgić et al., 2015; Lorusso et al., 2011; Vincent et al., 2009). Additionally, NA tree was constructed using N1 and N2 subtypes. All open-reading frames (ORFs) were aligned online using the MAFFT V.7 program (multiple sequence alignment program for Unix-like operating systems) (Katoh, Misawa, Kuma, & Miyata, 2002).

To elucidate the relation among sequenced viruses, phylogenetic trees for HA and NA were constructed using the JModelTest analysis (Posada & Modeltest, 2008) and a phylogenetic inference analysis was performed using the maximum-likelihood criterion. This analysis was applied to each sequence to identify the best substitution model, along with bootstrap analyses of the nucleotide sequence, to provide statistical support to the generated clades. A clade was considered a lineage only when bootstrap proportion was higher than 80%, corresponding to a probability >99%.

To estimate the evolutionary dynamics for each genomic segment, we implemented a time-scaled Bayesian method with a strict molecular clock. The phylogenetic construction was performed with the BEAST2 program, using the Hasegawa–Kishino–Yano substitution model (HKY) + gamma-distributed (G). Tree edition was performed with FIGTREE.

2.4 | Serum samples

A nationwide sampling programme was undertaken in 2014, including technical and semitechnical farms in all 32 Mexican states. A total of 21,000 swine serum samples were collected by direct puncture of the jugular vein with anticoagulant-free Vacutainer® tubes. All samples were labelled and taken to the FMVZ-UNAM; sera were obtained by centrifugation at 200 *g* for 15 min and kept at −20°C until processed. Among these samples, 14,550 came from the Central Bajío region in México, including the highest pork-producing states in the country (Jalisco, Guanajuato, Michoacán), Puebla, Hidalgo and México. Serum samples (1,472) from 86 counties at the states in this region, 10% of the total number of samples, were randomly chosen.

2.5 | Prevalence

To determine the circulation of the viral strains A/swine/Mexico/GtoDMZC01/2014 (H1N2) and A/swine/México/Mex51/2010 (H3N2) both isolated in the Central Bajío region, the presence of antibodies against these two swIAV strains was assessed by the hemagglutination inhibition (HI) test. Serum samples showing titres higher than 1:160 for the H1N2 strain were regarded as positive. H1N2-positive samples were selected to determine whether they contained antibodies capable of recognizing the field-isolated A/swine/México/Ver37/2010 (H1N1) strain. Those serum samples showing significantly higher anti-H1N1 titres than anti-H1N2 were regarded as H1N2 negative, to ensure the specificity of H1N2-positive results.

2.6 | Hemagglutination inhibition test

Hemagglutination inhibition tests were used to detect and titrate specific antibodies against the A/swine/México/GtoDMZC01/2014 (H1N2) viral isolate. Titres were compared with those obtained against the A/swine/México/Mex51/2010 (H3N2) virus strain. One hundred microlitres of each serum sample plus 50 μl of kaolin and 50 μl of 5% chicken erythrocytes were incubated at room temperature. Twenty-four hours later, once kaolin and erythrocytes settled, serum was extracted. Serum samples (50 μl) were serially diluted 1:2 (starting at an initial sample dilution of 1:4) and placed in a 96-well U-shaped plate (Nunc, Roskilde, Denmark) in 50 μl of PBS. Fifty microlitres of each virus was added to the samples, totalizing eight hemagglutinating units per well. Plates were then incubated for 60 min at room temperature, and 50 μl of 0.5% chicken erythrocytes was added. Results were recorded 1 hr later. Antibody titres were expressed as the maximal dilution at which the serum inhibited viral hemagglutination activity (Hernández et al., 1998; Ramírez, Carreón, Mercado, & Rodríguez, 1996). It is generally accepted that an HI titre of 1:40 against certain influenza virus strains correlates with an approximate protection rate of 50% against infections by homologous strains (Ng et al., 2013). Herein, HI titres higher than 1:160 against some virus strain were considered as positive for that strain and are probably related with an ongoing or a solved infection, although its precise meaning remains uncertain (Couch et al., 2013; Otani et al., 2016). Specific positive and negative sera were included in each assay. All experiments were carried out in an Animal Biosafety Level 3 (ABSL-3) laboratory.

3 | RESULTS

3.1 | Genetic characterization of H1N2 swIAV

The presence of all eight influenza gene segments in the isolated swIAV strain was evident after the third passage using the PathAmp™ FluA Kit (Life Technologies). All eight segments from the A/swine/México/GtoDMZC01/2014 (H1N2) isolate were sequenced, and the sequences were deposited in the GenBank database (Table 1). NCBI BLASTn was used to determine gene relatedness of each segment in the Guanajuato swIAV H1N2 isolate.

3.2 | Phylogenetic analysis of H1 and N2 genes

With respect to the evolutionary structure of the constructed topologies, the HA gene from the GtoDMZC01 isolate showed to be phylogenetically related to swine H1N2 viruses reported in United States. In this clade, hemagglutinin was found in H1N1 subtype swine viruses since 2005, and from the time-scaled phylogeny we estimated that the H1N2 GtoDMZC01 virus originated from a 2003 human H1N1-subtype (Figure 1). Other US swine H1N2 viruses, reported in 2000 and 2001, were found forming a separate branch along with the Mexican swine H1N1 strains isolated in 2012 and 2014, and with pandemic viruses, genetically similar to the A/swine/México/Ver37/2010 (H1N1) (GenBank Accession no.: CY122404) virus used by our team to identify by HI-specific antibodies against the herein reported H1N2 virus (Figure 1).

According to its phylogenetic relations, the NA gene from the A/swine/México/GtoDMZC01/2014 (H1N2) virus was most similar to the swine H3N2 from 2012 to 2013 reported in Mexico, with a possible ancestor of porcine origin. The NA gene of GtoDMZC01 was found in a different cluster from the Mexican swine H1N2 virus reported in 2011 and 2012. Finally, it was demonstrated that the virus herein reported differed from those strains reported in the United States in 2009 and 2010 (Figure 2).

3.3 | Genetic characterization of internal genes of A/swine/México/GtoDMZC01/2014 (H1N2)

The PB2 gene of the GtoDMZC01 isolate was of swine lineage, showing a maximum identity of 95% with other sequences

TABLE 1 GenBank accession numbers corresponding for each segment from the A/swine/México/GtoDMZC01/2014 (H1N2) strain

Segment	Gene	GenBank accession number
Segment 1	Polymerase PB2 (PB2)	KT225475
Segment 2	Polymerase PB1 (PB1 and non-functional PB1-F2 (PB1-F2)	KT225474
Segment 3	Polymerase PA (PA) and PA-X protein (PA-X)	KT225473
Segment 4	Hemagglutinin (HA)	KT225468
Segment 5	Nucleocapsid protein (NP)	KT225471
Segment 6	Neuraminidase (NA)	KT225470
Segment 7	Matrix protein 2 (M2) and matrix protein 1 (M1)	KT225469
Segment 8	Nuclear export protein (NEP) and non-structural protein 1 (NS1)	KT225472

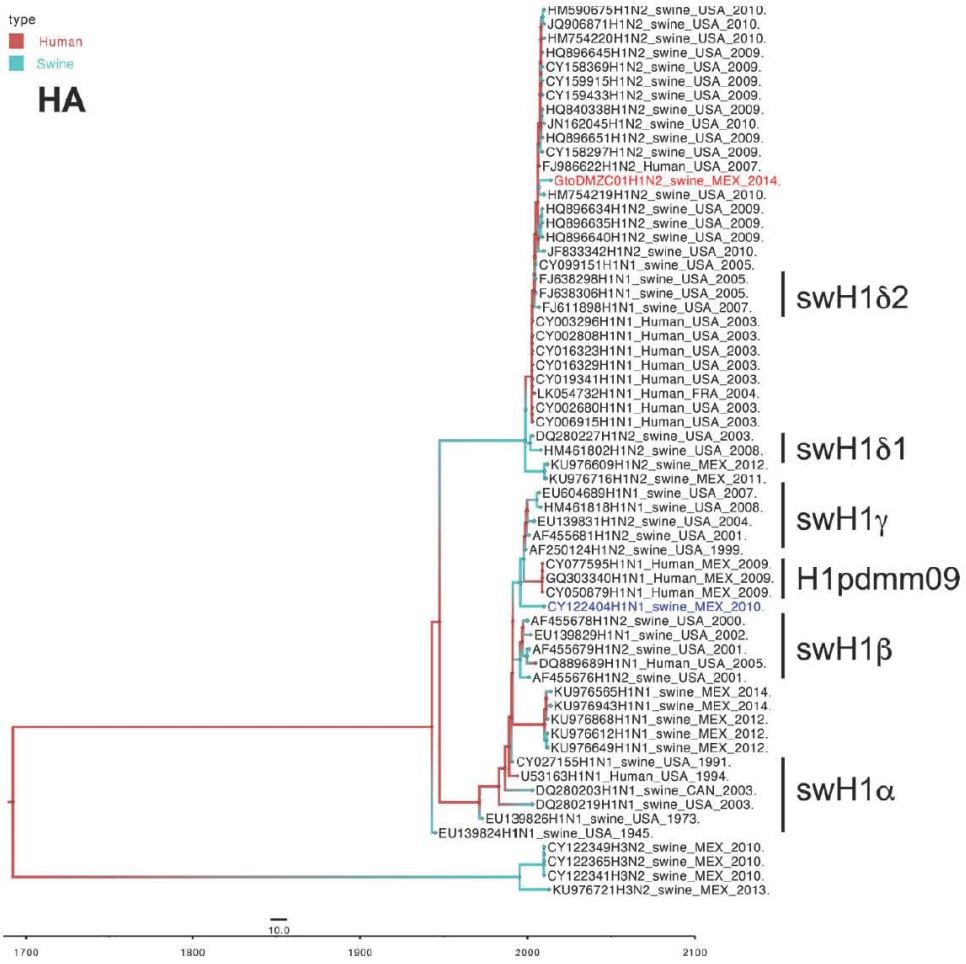

FIGURE 1 Molecular phylogenetic analysis by the Bayesian method using the HKY + G substitution model. Phylogenetic tree for the HA gene of the A/swine/México/GtoDMZC01/2014 (H1N2) virus (indicated by red) based on nucleotide sequences from the GenBank and sequences of the previously described H1 clusters: H1α, H1β, H1γ, H1δ1, H1δ2, H1pdm09 as indicated by the bars on the right of the tree. The blue colour indicate the H1N1 virus used to assess cross-reactions with H1N2

published in GenBank (Fig. S1-PB2). The PB1 gene showed a phylogenetic relation with Mexican H3N2-subtype viruses isolated in 2012-2013, possibly stemming from swine viruses (Fig. S1-PB1 gene). The PA gene showed a phylogenetic relation with the swine H1N1 and H3N2 subtypes isolated in Mexico in 2012 and 2013, with a possible human H1N1 lineage (Fig. S1-PA gene). The Mexican isolate also had NP genes from human origin and showed a phylogenetic relation with H1N1 subtypes isolated in 2012 and 2014, and with H3N2 subtypes isolated in Mexico in 2013, Fig. S1-NP gene). The M gene showed a phylogenetic relation with the Mexican H1N1- and H1N2-subtype viruses isolated in 2012, with a swine-originated ancestor (Fig. S1-M gene). The NS gene was of swine virus origin and showed a phylogenetic relation with the Mexican swine H1N1 subtype reported in 2012 and 2014 (Fig. S1-NS gene).

3.4 | Seroprevalence of H1N2 and H3N2 swine influenza virus in the Central Bajío region, México

3.4.1 | Seroprevalence by county

All sera used to assess the presence of specific antibodies against H1N2 swIAV were obtained from farms located in 86 counties at

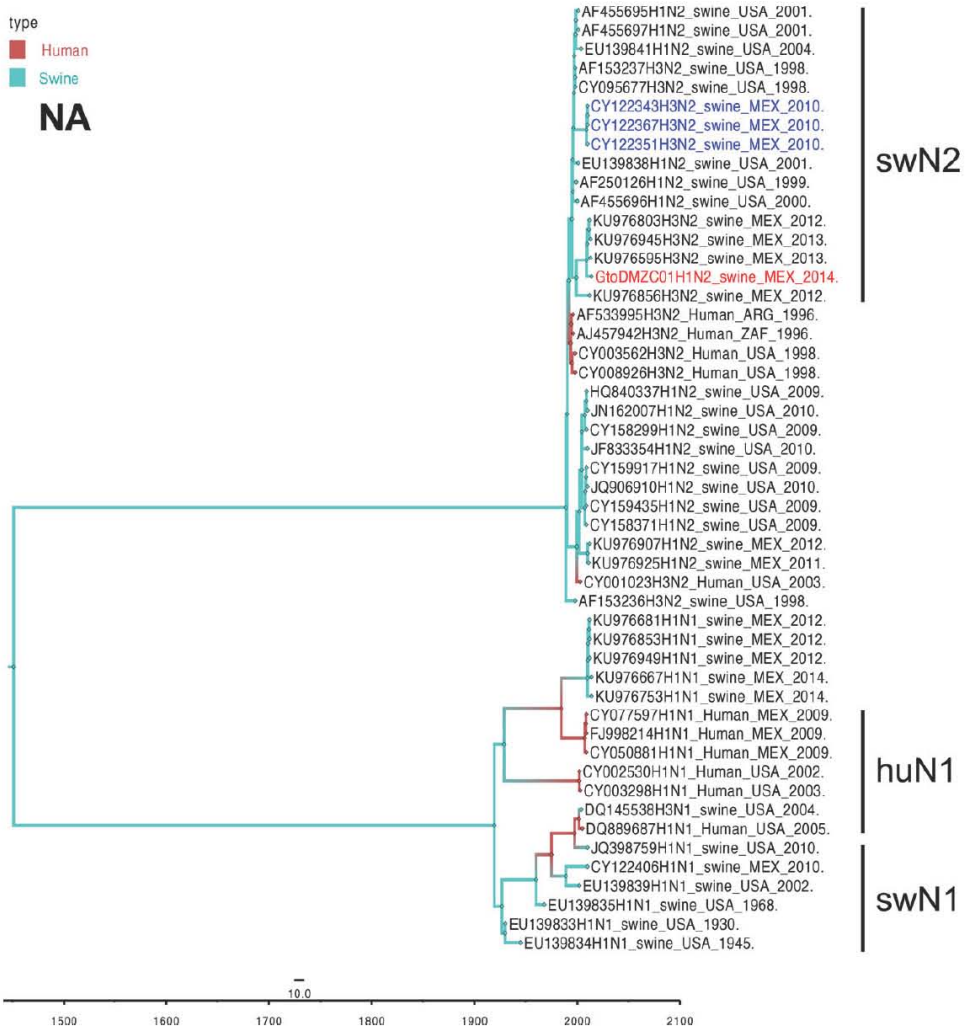

FIGURE 2 Time-scaled Bayesian tree inferred for the NA sequence of GtoDMZC01/2014 (H1N2) (indicated by red). NA phylogenetic tree with N1 and N2 subtypes and lineages indicated by bars on the right, and the H3N2 sequences used to evaluate seroprevalence in the Central Bajío region (indicated by blue)

the Central Bajio region in México. Similarly, the sera used to determine the presence of antibodies against H3N2 strains were obtained from farms located in 84 counties in the same region (Figure 3). A total of 1,472 HI tests were performed to assess the seroprevalence of A/swine/México/GtoDMZC01/2014 (H1N2) and A/swine/México/Mex51/2010 (H3N2) (GenBank Accession no.: CY122346:353) strains in serum samples from the region under study. The H3N2 viral strains showed the highest seroprevalence (54.76%), followed by H1N2 (26.74%) viruses (Table 2).

swIAV seroprevalence was assessed in 34 counties in Jalisco; 35.2% were positive to the H1N2 subtype and 64.7% were positive to H3N2. Twelve of the 34 sampled counties (35.3%) were positive to both strains (Figure 4a). The circulation of these two subtypes was evaluated in 23 counties of Guanajuato (50% of all counties in the state); specific anti-H1N2 antibodies were detected in 21.74% of studied counties, in contrast with the 56.52% of positivity detected for H3N2 (Table 3). Four counties (17.39%) in this state were positive to both strains (Figure 4b). Sera from 15

FIGURE 3 Central Bajio region in México, comprising the States of Jalisco, Guanajuato, Michoacán, Puebla, Hidalgo and State of México. The yellow area represents the six states covering the Central Bajío region in México, which represents one of the most important agricultural regions of the country

TABLE 2 Seroprevalence and total number of positive and negative counties for both Mexican swine influenza viruses in the Central Bajío region, México

	A/swine/México/GtoDMZC01/2014 (H1N2)		A/swine/México/Mex51/2010 (H3N2)	
N = 86/84	Negative	Positive	Negative	Positive
Region Central Bajío	63[a] 73.26%[b]	23 26.74%	38 45.24%	46 54.76%
Jalisco	22 64.7%	12 35.3%	12 35.3%	22 64.7%
Guanajuato	18 78.26%	5 21.74%	10 43.48%	13 56.52%
Michoacán	11 73.33%	4 26.67%	6 50%	6 50%
Hidalgo	3 75%	1 25%	1 25%	3 75%
Puebla	6 85.71%	1 14.29%	7 87.5%	1 12.5%
State of México	3 100%	0 0%	2 66.66%	1 33.33%

[a]No. of counties tested by state.
[b]Percentage values were calculated with respect to the number of farms sampled per state.

counties at Michoacán (13% of all counties in the state) were analysed for the H1N2 subtype, and sera from 12 counties were analysed for the H3N2 subtype; 26.67% of sampled counties were positive for H1N2 swIAV, and 50% of sampled counties were positive for H3N2 (Table 2). Only two counties in Michoacan were positive to both strains (Figure 4c). With respect to the states of Hidalgo (Figure 4d) and Puebla (Figure 4e), only one county in each state was positive for H1N2 (25% and 14.29% of sampled counties, respectively, Table 2). H3N2 seroprevalence was 75% in Hidalgo and 12.5% in Puebla. In Hidalgo, one municipality was detected positive to both influenza strains. No positive samples for H1N2 were detected in the state of México, and only one county showed a seroprevalence of 33.33% for the H3N2 subtype (Figure 4f).

3.5 | Seroprevalence of H1N2 and H3N2 swine influenza viruses in the Central Bajío region, as determined in sampled farms

As more than one farm could be found in one same county, the seroprevalence of the H1N2 and H3N2 subtypes per state was determined taking into account the number of sampled farms, disregarding its location within each county. In total, 184 farms from this region were sampled.

Specific antibodies against H3N2 swIAV were detected in 51.11% of farms in Jalisco; 27.78% (25 farms) were positive to H1N2, and 21.11% were double positive. In Guanajuato, prevalence was 30.77% for H3N2 and 15.38% for H1N2; 11.54% of farms were double positive. In Michoacan, 20% of farms were positive for H1N2 and 36% were positive for H3N2. Seroprevalence in Puebla was 11.11% for both subtypes. In Hidalgo, 20% and 60% of farms were positive for the H1N2 and H3N2 subtypes, respectively. Finally, the state of México showed positive results for H3N2 in 33.33% of sampled farms (Table 3).

3.6 | Specificity for detecting H1N2 subtype by HI

When two subtypes were present in a same sample, the one with highest titres showed higher specificity (Gaitán-Peredo, 2016; Saavedra-Montañez et al., 2013). Table 4 shows the titres of specific antibodies against H1N1 swIAV detected in all farms determined as only positive for H1N2, to assess the specificity of positive results. In Jalisco, 25 farms were positive for H1N2, and 44% of these farms showed higher levels of specific antibodies against this strain, while only 12% of farms in this state showed high anti-H1N1 antibodies; the remaining 44% of sampled farms showed similar antibody levels for both swIAV strains. In Guanajuato, 62.5% of all sampled farms showed higher levels of antibodies against the H1N2 strain. In contrast, one farm showed higher antibody levels against H1N1. Only one farm exhibited similar levels (1:160) for both strains. In Michoacan, 75% of sampled farms showed higher antibody titres for H1N2 than those recorded for H1N1. In Hidalgo, the highest antibody titres were specific for the H1N2 swIAV strain.

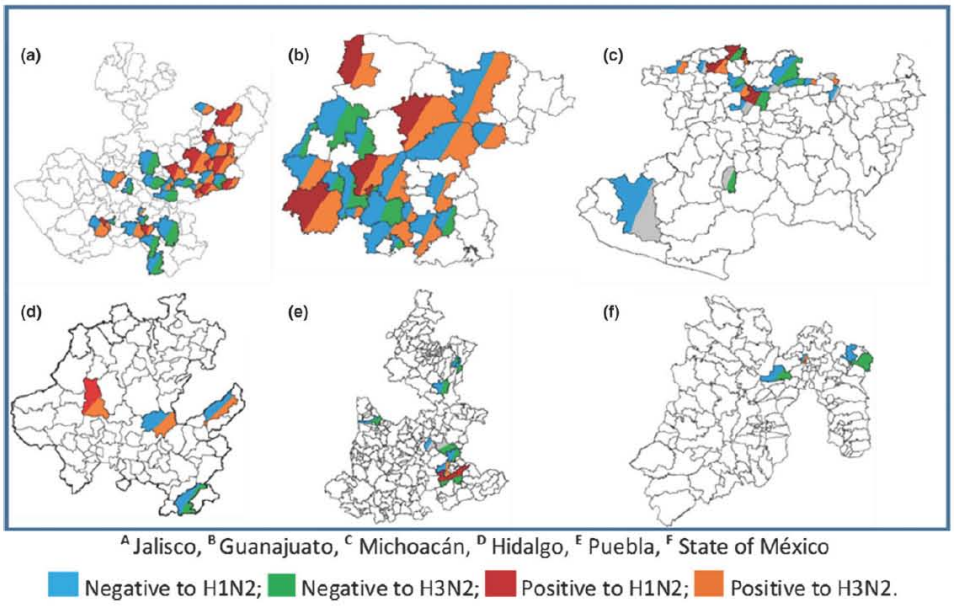

FIGURE 4 Shown separately the states that comprise the Central Bajío region in México. (a) Jalisco, (b) Guanajuato, (c) Michoacán, (d) Hidalgo, (e) Puebla, (f) State of México, divided into counties. The blue represents the counties negative to H1N2 strain; green represents the counties negative to the H3N2 strain; red represents counties positive to H1N2 strain, and the orange represents counties positive to the H3N2 strain

TABLE 3 Swine farms positive against each viral subtype

N = 184	A/swine/México/ GtoDMZC01/2014 (H1N2)	A/swine/México/ Mex51/2010 (H3N2)	Double-positive Farms
Jalisco (90)	25[a] (27.78%)	46 (51.11%)	17 (21.11%)
Guanajuato (52)	3 (15.38%)	16 (30.77%)	6 (11.54%)
Michoacán (25)	5 (20%)	9 (36.0%)	0 (0%)
Puebla (9)	1 (11.11%)	1 (11.11%)	0 (0%)
Hidalgo (5)	1 (20%)	3 (60.0%)	1 (20.0%)
State of México (3)	0 (0%)	1 (33.33%)	0 (0%)

[a]Number of positive farms analysed by state.

4 | DISCUSSION

The Central Bajio region is one of the main geographic areas in the Mexican agroindustry, as four of the top pork-producing states are located there (Jalisco, Puebla, Guanajuato and Michoacan); the region also includes the states of Hidalgo and Mexico. When analysing the genetic characteristics of influenza viruses, several studies have demonstrated that HA is the main viral gene involved in host specificity. However, internal genes can also play a significant role in host exclusion (Webster, Bean, Gorman, Chambers, & Kawaoka, 1992). According to what is shown on Figure 1, the HA segment from the A/swine/México/GtoDMZC01/2014 (H1N2) virus demonstrated to be similar to other H1N2 sequences reported in previous years, similar to the delta 2 viruses found in North America (H1δ2). This GtoDMZC01 isolate is relevant because it has been demonstrated that its H1 has a different origin to other swine Mexican H1N2 viruses reported in 2011–2013 (Nelson et al., 2015).

The phylogenetic relation of neuraminidase (N2) showed that this virus is phylogenetically related to the Mexican swine H3N2 subtype reported from 2012 to 2013; however, it is closer to the H1N2 strains reported in the United States in 1999–2001 than to the Mexican H1N2 viruses reported in 2011 and 2012. In contrast to its N2 swine lineage, the HA of GtoDMZC01 virus is derived from the human lineage, this is due to the segmented nature of the genome of influenza viruses, which could undergo a reassortment of such segments during replication, either with virus of differing origin or of differing subtype (Carrat & Flahault, 2007; Treanor, 2004).

Genetic characterization of the PB2 gene from the A/swine/México/GtoDMZC01/2014 (H1N2) virus showed that PB2 was of swine origin, which has been conserved among swine influenza strains by the last 18 years, at least. On the other hand, no significant similitude percentages were found with PB1 genes from

TABLE 4 Antibody titres against A/swine/México/GtoDMZC01/2014 (H1N2) and A/swine/México/Ver37/2010 (H1N1)

	A/swine/México/GtoDMZC01/2014 (H1N2)	A/swine/México/Ver37/2010 (H1N1)
Jalisco		
Farm 1	1:160[a]	Negative
Farm 2	1:640	1:640
Farm 3	1:320	1:160
Farm 4	1:160	1:80
Farm 5	1:640	1:640
Farm 6	1:160	1:640[a]
Farm 7	1:320[a]	1:80
Farm 8	1:640[a]	1:160
Farm 9	1:320[a]	1:80
Farm 10	1:160	1:320
Farm 11	1:640[a]	1:80
Farm 12	1:320[a]	Negative
Farm 13	1:640[a]	Negative
Farm 14	1:160	1:1,280[a]
Farm 15	1:1,280[a]	1:160
Farm 16	1:160[a]	Negative
Farm 17	1:320	1:320
Farm 18	1:160	1:80
Farm 19	1:160	1:80
Farm 20	1:1,280[a]	1:80
Farm 21	1:160	1:80
Farm 22	1:160	1:1,280[a]
Farm 23	1:160	1:160
Farm 24	1:160	1:160
Farm 25	1:640[a]	1:80
Total farms with higher titres	11 (44%)	3 (12%)
Guanajuato		
Farm 1	1:320	1:1,280[a]
Farm 2	1:320	1:640
Farm 3	1:640[a]	1:160
Farm 4	1:160[a]	Negative
Farm 5	1:160[a]	Negative
Farm 6	1:1,280[a]	Negative
Farm 7	1:1,280[a]	1:320
Farm 8	1:160	1:160
Total farms with higher titres	5 (62.5%)	1 (12.5%)
Michoacan		
Farm 1	1:160[a]	Negative
Farm 2	1:320	1:160
Farm 3	1:160[a]	Negative
Farm 4	1:160[a]	Negative
Total farms with higher titres	3 (75%)	0 (0%)
Hidalgo		
Farm 1	1:1,280[a]	Negative
Total farms with higher titres	1 (100%)	0 (0%)
Puebla		
Farm 1	1:640	1:1,280
Total farms with higher titres	0 (0%)	0 (0%)

[a]Farms with higher HI titres was considered, when it exceeded at least twofold dilutions with respect to subtype H1N1.

previously reported H1N2 strains, which indicates that the PB1 gene in the GtoDMZC01 strain resulted from the recombination of different subtype strains. The PA gene found in the new Mexican H1N2 virus herein reported showed a high similarity percentage with PA genes from different subtypes, and thus, it is very likely to come from H3N2- and H1N1-subtype swine viruses reported in Mexico.

As the NP gene plays an important role in determining the viral species specificity (Scholtissek, Bürger, Kistner, & Shortridge, 1985), the phylogeny of the NP gene was completely characterized with identity levels of 97%–100% in BLASTn. This analysis suggests a possible evolutive divergence from human viruses of pandemic lineage. Therefore, this is the first report of a H1N2 subtype with a NP gene from pandemic viruses. It is interesting to note that in experimental models seeking to adapt A (H1N1) pdm09 viruses to mice, genetic analysis showed the presence of internal genes from pandemic viruses in those strains with significantly higher virulence and pathogenicity in mice; NP is among these genes, with only an amino acid substitution (I353V) (Otte et al., 2015; Prokopyeva, Sobolev, Prokopyev, & Shestopalov, 2016). This should be taken into account, as naturally occurring amino acid modifications could cause an increase in the virulence and pathogenicity of this virus.

Our serology results confirm that the H3N2 swIAV strain is currently circulating in the Central Bajío region, México, as it was present in all of the evaluated states, and high antibody titres for this strain were found in 54.76% of all sampled counties. On the other hand, while seropositive samples for the new H1N2 strain were found in 5 of 6 states under study (83% of sampled states), H1N2 seroprevalence per county was significantly lower than H3N2 seroprevalence, as positive titres were found in only 26.74% of counties in the region. According to preliminary data on swine population published by SIAP, Jalisco was the Mexican state with the highest swine population in 2014; high anti-H1N2 antibody titres were detected in 35.30% of all sampled counties. By analysing the geographic location of high-titre counties, it is clear that they are grouped in a region of the state (Figure 4a), limiting with Guanajuato (Figure 4b); the latter showed a seropositivity of 21.74% in the

sampled counties. These two states showed the highest seropositivity percentage in the Central Bajío region per number of counties. Additionally, considering the circulation of the H1N2 and H3N2 strains in one same county, it is noticeable that 35.3% of all Jalisco counties and 17.39% of Guanajuato counties showed a simultaneous circulation of both viral strains, demonstrating their wide circulation.

On the other hand, the H1N1 swIAV strain is also circulating widely in Mexico. Thus, to prevent cross-recognition in the HI, all H1N2-positive samples were analysed in parallel against the A/swine/México/Ver37/2011 (H1N1) strain. Thus, it was possible to consider the specificity of antibody detection. This analysis was performed on a per-farm basis instead of per-county, as the presence of a positive farm in a given county was sufficient to consider as positive the whole county; thus, the farm with twofold increase in HI titres for one of the two viral strains was recorded with specific seropositivity for that strain. Thus, 44% of all Jalisco farms were H1N2 positive, while only 12% of farms were H1N1 positive. High prevalence rates for H1N2 (62.5%) and H1N1 (12.5%) were detected in Guanajuato. Three of the four farms in Michoacan showed higher anti-H1N2 antibody levels than anti-H1N1 (Table 4). All four farms in this state are located in counties bordering Jalisco and Guanajuato (Figure 4), evidencing the circulation of the new influenza strain in this geographic region.

The seroprevalence study herein reported evidences the circulation of a new H1N2 swine influenza virus in the states of the Central Bajío region in México. It is clear that the main region of virus circulation is the bordering area of Jalisco, Guanajuato and Michoacan. Although with lower numbers of positive sera, there is evidence of virus circulation in Hidalgo and Puebla as well, with high titres of specific antibodies against this swIAV strain. This suggests that the geographic proximity and exchange of animals among these states has favoured virus dissemination throughout the region.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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11. ANEXOS

11.1 Principio del ensayo de qRT-PCR

La qRT-PCR es una herramienta de diagnóstico molecular sensible y específica. Este sistema, se basa en la amplificación selectiva de un segmento por PCR y su detección específica mediante una sonda TaqMan, que al hibridar con el producto de amplificación, es degradado generando fluorescencia en el momento que ocurre la amplificación (en tiempo real). Esta fluorescencia es detectada por un equipo termociclador de tiempo real, que posee un sistema óptico de excitación y de detección de fluorescencia. Debido a que el genoma del virus de Influenza es de ARN, es necesario para su identificación obtener primero el ARN de la muestra, generar una copia de ADN (cDNA) y posteriormente amplificarla y detectarla por qRT-PCR. En la detección selectiva de un segmento diagnóstico del virus de Influenza, se emplea una sonda TaqMan, que es un oligonucleótido sintético que posee la secuencia de nucleótidos complementaria al producto de amplificación. Esta sonda TaqMan posee dos fluoróforos que al estar en los extremos del oligonucleótido, no generan fluorescencia ya que se inhiben mutuamente. Cuando la sonda TaqMan hibrida específicamente al producto de amplificación, esta es degradada por la acción 5' exonucleasa de la ADN Taq polimerasa. Cuando esto sucede, se separa el fluoróforo reportero de otro que inhibe su fluorescencia. Solamente cuando la sonda hibrida con el segmento de ADN específico y es degradada, es posible detectar la amplificación del segmento de ADN diagnóstico. Esto hace la detección sumamente específica y sensible.

11.2 Procedimiento de la qRT-PCR para el virus de influenza tipo A

1.- Añada hasta 10 µl de la muestra de RNA o del control positivo en un tubo. Cuando sea necesario complemente a un volumen de 10 µl con H_2O DEPC.

2.- Añada 10 µl de la Super Mix RT-PCR-TR 2X

3.- Someta el tubo a amplificación-detección en un equipo termociclador de Tiempo Real.

Condiciones para el termociclador.

42°C por 30min, 95°C por 10min, 40 ciclos de 95°C por 15seg y 60°C por 45seg.

Reactivo RNAGT

El reactivo RNAGT es una solución para la purificación de RNA en un solo paso. Este reactivo está basado en el método de P. Chonczynski y N. Sacchi (Anal. Biochem. 1987, 162:156-159), que emplea detergentes, agentes reductores y tiocianato de de guanidina, para disgregar y solubilizar eficientemente macromoléculas orgánicas, y fenol ácido, que atrapa el ADN y proteínas en las fase orgánica. Este reactivo es la formulación más ampliamente usado en la obtención de RNA de una forma rápida y económica.

Procedimiento para la purificación de RNA con el reactivo RNAGT

1. A la muestra de tejido (0.5 gr) o líquido (500ul) agregar 1 ml de RNAGT homogeneizar vigorosamente (vortex) por 15 segundos.
2. Adicionar 200 µl de cloroformo, agitar vigorosamente por 15 segundos (vortex).
3. Incubar a temperatura ambiente por 5 minutos.
4. Centrifugar a 10,000 g por 15 min a 4°C.
5. Recuperar la fase acuosa en un tubo eppendorf de 1.5 ml y adicionar 10 µg de glicógeno (opcional), mezclar.
6. Adicionar 1 volumen de isopropanol absoluto frío, homogenizar por inversión.
7. Incubar a -20°C por una hora (opcional).
8. Centrifugar 10,000 g por 10 min a 4ºC.
9. Decantar y adicionar a la pastilla 500 µl de etanol al 75% en H_2O DEPC, mezclar.
10. Centrifugar 10,000 g por 5 min a 4ºC.
11. Decantar cuidadosamente y secar la pastilla al vacío a temperatura ambiente.
12. Resuspender la pastilla en H_2O DEPC (aprox. 25 ul)
13. Almacenar a -70°C.

11.3 Flujo de trabajo de la secuenciación del virus de influenza

El primer paso fue generar una librería de fragmentos de DNA flanqueados por adaptadores en el Ion Torrent, esto se puede hacer mediante la ligación de los adaptadores a los productos de PCR o por la adición de las secuencias adaptadoras durante el PCR mediante el diseño de cebadores de con las secuencias adaptadoras de iones en el extremo 5′. Los fragmentos de la biblioteca son entonces clonados y amplificados en las partículas esféricas del Ion Torrent. La amplificación clonal se lleva a cabo por PCR en emulsión, las partículas esféricas recubiertas por plantillas son aplicadas en los pozos del chip del Ion Torrent con una corta centrifugación. El chip es colocado en el PGM y en la pantalla táctil se configura la corrida de secuenciación. Una vez que los datos se generan en el secuenciador Ion Torrent es automáticamente transferido al servidor del equipo. Aquí los datos se ejecutan a través del procesamiento de señales y la base de algoritmos que producen las secuencias de DNA asociados con lecturas individuales.

Kits y protocolos empleados en el procedimiento de secuenciación masiva:

Extracción de RNA viral específico. QIAamp® MinElute® Virus Spin.

Mezcla de reacción. One Step RT-PCR Kit Quiagen®.

Purificación de producto de PCR. QUIAquick Gel Extraction Kit.No. de Catálogo 28704.

Cuantificación productos. Equipo: Nanodrop 2000 Thermo Scientific.

Reacción de secuenciación. BigDye® Terminator v3.1 Cycle Sequencing Kits.

Purificación reacción de secuencia. Centri Sep Spin Columns No de Catalogo CS-901.

Lectura de secuencia. Equipo: Analizador Genético Ion Torrent.

Edición y alineación de secuencia. BioEdit Sequence Alignment Editor Copyright 1997-2005 Tam Hall.

Búsqueda de regiones similares de la secuencia editada. National Center for Biotechnology Information. www.ncbi.nlm.nih.gov Basic Local Alignment Search Tool (BLAST).

Alineación y construcción de árbol filogenético. Clone Manager Professional Suite, Clone Manager 7, versión 7.0, Align Plus 5 versión 5.0, Primer Designer 5, versión 5.0

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