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Genetic changes detected in the internal genes of porcine influenza viruses isolated in Mexico

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Abstract

To date, only one report has been published in Mexico detailing the genetic changes and evolutionary divergence in the so-called internal genes basic polymerase-one (PB1), basic polymerase-two (PB2), polymerase acid (PA), nucleoprotein (NP), matrix protein (M), and non-structural protein (NS) of the swine influenza virus that circulated during the 2009 pandemic and in 2010. The aim of this study was to evaluate the evolutionary divergence and genetic changes in these internal genes of the swine influenza virus strains isolated in Mexico in 2009-2010. To characterize the evolutionary history and the phylogenetic relationship among the isolated virus strains, a Maximum Likelihood Model Test analysis and a phylogenetic bootstrap test with 1000 replicates were performed. The phylogenetic relationships among the PB1, PB2, PA, NP, M and NS genes correspond to similar swine virus isolates reported in other countries. However, the PA gene from the A/swine/Mexico/Qro35/2010 (H1N1) virus is closely related to a H3N2-subtype human virus. The PB2, NP, and M genes of the Mexican swine influenza viruses that circulated during 2009 and 2010 maintained the distribution of the Triple Reassortant Internal Genes (TRIG). Significantly, our study demonstrates the presence of a human H3N2 virus PA gene in the A/swine/Mexico/Qro35/2010 (H1N1) virus.

Keywords: Swine influenza virus, Phylogenetic characterization, Internal genes, Mexico.

Introduction

The genome of the influenza A virus can be characterized as a single RNA strand segmented into eight pieces. These segments encode twelve proteins: basic polymerase-two (PB2), encoded by segment 1; PB1, PB1-F2, and N40, encoded by segment 2; polymerase acid (PA), encoded by segment 3; hemagglutinin (HA), encoded by segment 4; nucleoprotein (NP), encoded by segment 5; neuraminidase (NA), encoded by segment 6; matrix protein M1 and ion channel M2, encoded by segment 7; and finally, two nonstructural proteins (NS1 and NS2), encoded by segment 8. In total, 17 HA (Tong *et al.*, 2013) and 9 NA variant proteins have been identified thus far, and they provide the basis for classifying the type A influenza virus into viral subtypes (Vincent *et al.*, 2008).

Influenza viruses are among the most important human pathogens because they cause seasonal epidemics and occasional pandemics. Three major influenza pandemics were recorded in the last century: in 1918, 1957, and 1968. In March and early April 2009, the first cases of influenza A/H1N1pdm09 were detected in Mexico and in the United States. Because it is easily transmitted among humans, the virus spread rapidly around the world, causing the first pandemic flu of the 21st century (Dawood *et al.*, 2009). Genetic characterization and phylogenetic analysis showed that the pandemic strain was of swine origin. The PB1, PB2, PA, HA, NP, and NS genes characterized from the isolates were found to have been derived from a North American triple reassortant swine virus isolate, whereas the NA and M genes were found to have originated from a Eurasian-lineage swine virus (Garten *et al.*, 2009).

The genetic rearrangement of influenza viruses that infect birds, humans, and pigs has been documented in pig hosts. After transmission to pigs, the avian and human viruses undergo divergent evolution, and new genetic lines are established (Vincent *et al.*, 2009; Karasin *et al.*, 2002; Webby *et al.*, 2004).

The double reassortant H3N2 virus was first isolated from pigs during a severe influenza-like disease in North Carolina farms in 1998. Genetic analysis of that virus revealed that it contained segments from the classic swine lineage (NS, NP, M, PB2, and PA) along with some segments from a circulating human influenza virus (HA, NA, and PB1). Subsequently, a triple reassortant H3N2 virus was isolated from pigs in Minnesota, Iowa, and Texas and was found to contain gene segments from the classical swine virus (NS, NP, and M), human virus (HA, NA, and PB1), and avian virus (PB2 and PA) (Karasin *et al.*, 2006). A constant feature in this new re-association among different virus types is the presence of the specific internal genes PB1, PB2, PA, NP, M, and NS, which are collectively known as the Triple Reassortant Internal Genes (TRIG) originally derived from a triple reassortant H3N2 virus.

Since 2005, a number of human-like H1 viruses, which are genetically and antigenically distinct lineages derived from classic swine H1 virus, have been identified in Canadian pigs and have disseminated throughout the United States (Zhou *et al.*, 1999; Gramer, 2008). The six internal genes in human H1 virus seem to be similar to those found in the TRIG set in the contemporary virus isolates. These findings suggest that viruses containing the TRIG set are able to accept multiple types of HA and NA genes, which in turn may provide a selective advantage to the swine viruses which carry them.

Only one report has been published in Mexico on a porcine viral isolate closely related to the A/H1N1 pdm09 virus (Escalera *et al.*, 2012). Furthermore, no studies on the genetic changes and evolutionary divergence of the internal genes PB2, PB1, PA, NP, M and NS1 in the swine influenza viruses that circulated in the 2009 pandemic and in 2010 have been reported. Therefore, the aim of this study was to identify the changes in these genes and delineate their evolutionary history.

Materials and methods

Sample collection, viral isolation, and RNA extraction

Lung tissue samples from recently slaughtered pigs were collected in 2009 and 2010 at slaughterhouses in eighteen states in Mexico for a previous study and kept refrigerated. RT-PCR and viral isolation were carried out in a biosafety level 3 (BSL3)

laboratory in the Department of Medicine and Husbandry of Pigs (DMZC), Facultad de Medicina Veterinaria y Zootecnia (FMVZ) de la Universidad Nacional Autónoma de Mexico (UNAM).

Pigs from 7 out of 123 inspected farms (5.6%) tested positive for the viral M gene. RT-PCR was performed using a OneStep RT-PCR kit from QIAGEN® following the manufacturer's instructions and the primers SIVM-F (5´TGAGTCTTCTAACCGAG-GT 3´) and SIVM-R (5´ AGCGTCTACGCTGCAGTC R). The cycling conditions for gene amplification were as follows: one cycle at 50 °C for 30 minutes, one cycle at 95 ° for 15 minutes, 35 cycles at 94 °C for 30 seconds, 60 °C for 60 seconds and 72 °C for 60 seconds and a final cycle at 72 °C for 10 minutes, leaving the sample at 4 ° indefinitely. Once the PCR product was obtained, 5 µl of the PCR product was mixed with 2 µl of sample buffer and resolved in a 2% agarose gel at 90 volts for 50 minutes in a horizontal electrophoresis chamber. The gel was then stained with ethidium bromide, and the expected amplicons were visualized on a transilluminator Bio Imaging System and identified using an "imaging system Gel Logic 112" Kodak imager and the pUC Mix 8® molecular weight marker.

For viral replication, a 200-µl virus aliquot was inoculated under sterile conditions in the allantoic cavity of 9- to 11-day-old ALPES1® specific pathogen free (SPF) chicken embryos and incubated at 37° C. Allantoic fluid was collected at 24, 48, and 72 hours after inoculation and centrifuged at 3,500 rpm for 5 min. Virus titers were assessed by hemagglutination assay. Viral RNA was extracted using a PureLink™ Viral RNA/DNA kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. After extracting RNA from seven virus isolates, the full genomic sequence of all the isolates was determined using an Illumina sequencing platform.

Sequencing

Whole-genome sequencing was performed at the Instituto de Biotecnología (UNAM). Samples from two hundred base-pair-sized libraries, prepared using an Illumina mRNA-Seq 8 sample prep kit according to the manufacturer's instructions, were loaded in separate lanes of a flow cell of a Genome Analyzer IIx (Illumina, San Diego, CA). Sequencing was performed by 36 cycles of single-base pair extension. For image analysis, a Genome Analyzer Pipeline version 1.4 was used. Viral reads were assembled with MAQ version 0.7.1 (Mapping and Assembly with Qualities) (Li *et al.*, 2008), using the influenza A isolate A/Netherlands/602/2009 as the assembly reference genome. Coverage graphics were generated using R2.12 (Team, 2010). Only reads matching the reference genome were considered for further analysis. Between 7.6 and 10.5 million 36-nt-long reads were obtained from each sample. Over 99.3% of the viral genome was sequenced with a 120x to 1006x coverage. All reported sequences were submitted to GenBank and are available at the National Center for Biotechnology (NCBI) (Table 1).

Phylogenetic analysis

Once the consensus sequences were obtained, a search for identity and greater than 95% coverage was performed using NCBI BLASTn (Basic Local Alignment Search Tool). The resulting sequences were compared with other sequences in GenBank for subsequent phylogenetic reconstruction.

Table 1. Genotypic characterization of porcine isolates.

Isolate	Sample	Nomenclature	Access GenBank	Subtype
1	Lung, trachea, tonsil.	(A/swine/Mexico/Mex19/2010(H1N1))	PB2: CY122330 PB1: CY122331.1 PA: CY122332 NP: CY122334 M: CY122336 NS:CY122337	H1N1
2	Lung	(A/swine/Mexico/Ver29/2010(H1N1))	PB2: CY122386 PB1: CY122387 PA: CY122388 NP: CY122390 M: CY122392 NS:CY122393	H1N1
3	Lung	(A/swine/Mexico/Qro35/2010(H1N1))	PB2: CY122378 PB1: CY122379 PA: CY122380.1 NP: CY122382 M: CY122384 NS: CY122385	H1N1
4	Lung	(A/swine/Mexico/Ver37/2010(H1N1))	PB2: CY122401 PB1: CY122402 PA: CY122403 NP: CY122405 M: CY122407 NS: CY122408	H1N1
5	Lung	(A/swine/Mexico/Mich40/2010(H3N2))	PB2: CY122362 PB1:CY122363 PA: CY122364 NP: CY122366 M: CY122368 NS:CY122369	H3N2
6	Lung	(A/swine/Mexico/Mex51/2010(H3N2))	PB2: CY122346 PB1: CY122347 PA: CY122348 NP: CY122350 M: CY122352 NS: 122353	H3N2
7	Semen	(A/swine/Mexico/Mex52/2010(H1N1))	PB2: CY122354 PB1: CY122355 PA: CY122356 NP: CY122358 M: CY122360 NS: CY122361	H1N1

The reference sequences for pandemic viruses were A/Mexico/InDRE4487/2009 (H1N1), A/Mexico/LaGloria-3/2009 (H1N1), and A/MexicoCity/005/2009 (H1N1). The sequences used are shown in Table 2.

The sequence of the A/Mexico/InDRE4487/2009 (H1N1) virus strain was chosen as the reference because it was reported by the Institute of Epidemiological Diagnosis and Reference (InDRE), the accredited body for epidemiological surveillance of the National Network of Public Health Laboratories (RNLSP) in Mexico. The A/Mexico/LaGloria-3/2009 (H1N1) virus strain was selected as one of the isolates from "ground zero" that led to the 2009 pandemic. Finally, the A/Mexi-

Table 2. Genotypic characterization of pandemic viruses.

Isolate	Nomenclature NCBI	GenBank Accession	Subtype
1	A/Mexico/InDRE4487/2009(H1N1)	PB2:FJ998206 PB1: FJ998226 PA: FJ998223 NP: FJ998217 M: FJ998211 NS: FJ998220	H1N1
2	A/Mexico/LaGloria-3/2009(H1N1)	PB2:CY077592.1 PB1: PA: CY077594 NP: CY077596 M: CY077598 NS: CY077599	H1N1
3	A/MexicoCity/005/2009(H1N1)	PB2: CY050886 PB1: CY050885 PA: CY050884 NP: CY050882 M: CY050880 NS: CY050883	H1N1

coCity/005/2009 (H1N1) virus strain was included because it was reported by the Influenza Genome Sequencing Project (IGSP). The inclusion of viral sequences registered in Mexico may help determine whether these porcine isolates share a common ancestral origin with the pandemic viruses in Mexico.

The sequences for each gene were progressively aligned online using MAFFT V.7 (multiple alignment program for amino acid or nucleotide sequences) (Kazutaka *et al.,* 2002).

To elucidate the phylogenetic relationship among the sequenced viral genomes, the JModelTest analysis (Posadas D., 2008) along with the criterion of Maximum Likelihood (ML) was used. The algorithm was applied to each set of sequences to determine the best substitution model along with a bootstrap phylogenetic test set of 1000 replicates to provide statistical support for each branch generated. Only bootstrap values greater than or equal to 0.80 (80%) were considered relevant. ML-inferred trees for the nucleotide sequence of each gene were constructed using different substitution models mainly using the Bayesian information criteria (BIC):

- PB1 Gene: Hasegawa-Kishino-Yano (HKY) + Gamma Distributed (G)
- PB2 Gene: Hasegawa-Kishino-Yano (HKY) + Gamma Distributed (G)
- PA Gene: General Time Reversible (GTR) + Gamma Distributed (G)
- NP Gene: Hasegawa-Kishino-Yano (HKY) + Gamma Distributed (G)
- M Gene: Kimura 2-parameter (K2) + Gamma Distributed (G)
- NS Gene: Hasegawa-Kishino-Yano (HKY) + Gamma Distributed (G)

MEGA (Molecular Evolutionary Genetics Analysis) software version 5.2 (Tamura *et al.,* 2011) was used in the bioinformatic analysis of PB1 and NS sequences. Phylogenetic trees were built from nucleotide sequences using the Bayesian criterion. For both sequences, a phylogenetic tree was generated using the GTR model.

Results and discussion

Phylogenetic characterization of internal genes PB2, PB1, PA, NP, M and NS

With respect to the evolutionary structure of the constructed topologies for the PB2, NP, and M genes, pandemic viruses were grouped in clusters that were totally different from the transient changes common to all swine viruses included in the analysis. This analysis showed that the PA gene of all pandemic viruses analyzed was inherited from a common ancestor of avian origin and diverged in swine, thereby maintaining a line of continuous evolution (Fig. 1-6).

PB2 gene

Analysis of the PB2 gene (segment 1) demonstrated that all of the isolated viruses were distributed along a specific gene branch of porcine origin after evolutionary divergence between birds and pigs and are phylogenetically related to A/swine/Minnesota/1192/2001 (H1N2). Isolates 29, 35, 37, and 52 (H1N1), as well as 19 (H1N1), 40, and 51 (H3N2), diverged from the same ancestor but under different evolutionary lines. Because they are different viral subtypes, isolates 19, 40, and 51 share a synapomorphic trait (Fig. 1).

Six total amino acid changes that are unique for the pandemic viruses with respect to all examined sequences were identified in the PB2 gene. Isolates 29, 35, 37, and 52 showed four amino acid changes at positions I292M, V386A, V731I, and L434F. Two of these changes were found exclusively in these viruses among all aligned sequences, including pandemic viruses. Position 292 of the PB2 protein matched with the A/swine/Illinois/SG1141/2003 (H1N1) and A/swine/Korea/JNS06/2004 (H3N2) viruses, and the amino acid in position 731 is found in the aforementioned viruses and in A/swine/Minnesota/1192/2001 (H1N2). Moreover, isolates 19, 40 and 51 displayed four single amino acid changes (not present in any other sequence) at positions A395V, I451T, L475M, and T637A (Table 3).

PB1 gene

Phylogenetically, isolates 19, 29, 35, 37, and 52 (H1N1) were included in a monophyletic group with a synapomorphic trait originating from the American virus A/swine/Nebraska/00188/2003 (H3N2) of different subtype, whereas isolates 40 and 51 (H3N2) were grouped in a separate cluster (Fig. 2).

Eleven amino acid changes with respect to the other sequences were observed in the PB1 gene isolated from pandemic viruses. Isolates 19, 29, 35, 37, and 52 (H1N1) were found to have the identical amino acid mutations K214R, I368V, D464N, T469A, and F574Y; they also had M744I in common with the A/swine/Nebraska/00188/2003 (H3N2) virus. Isolates 40 and 51 (H3N2) were the only sequences with the D565N mutation (Table 3).

PA gene

The phylogenetic relationship of the PA gene (segment 3) from A/swine/Mexico/Qro35/2010 (H1N1) with the human seasonal influenza viruses allowed us to identify heterogeneous evolutionary branches within a single monophyletic group and a close relationship to the human virus subtype H3N2 with a bootstrap support value of 100%. The PA gene sequences from the other isolates analyzed were found to be evolutionarily and recursively related to A/swine/Minnesota/1192/2001 (H1N2).

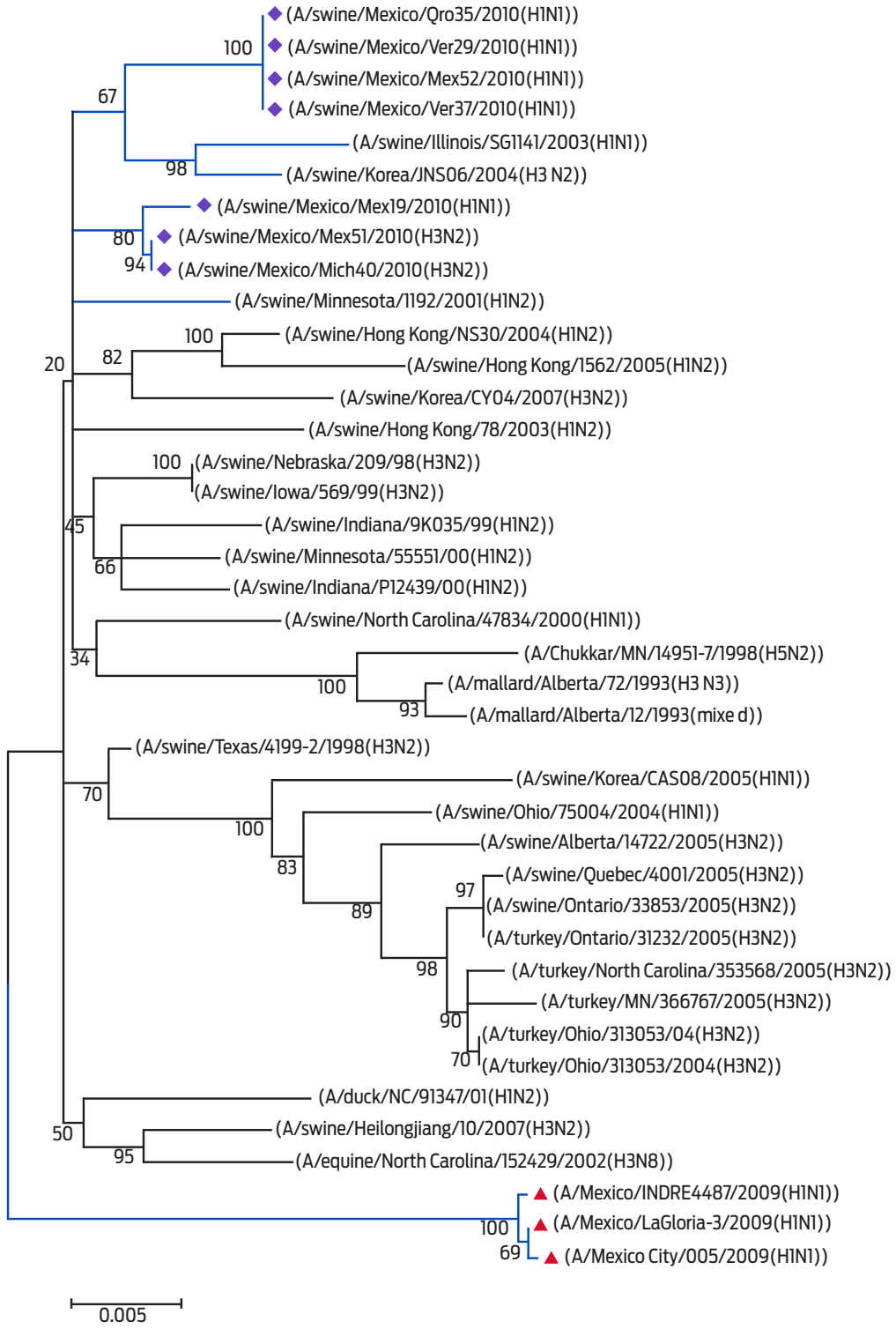

Figure 1. Molecular phylogenetic analysis by the Maximum Likelihood (ML) method using the HKY + G substitution model. Phylogenetic tree of the PB2 gene of the seven viral isolates (violet diamonds) and the pandemic virus (red triangles) based on nucleotide sequences and other sequences in GenBank. The tree is drawn to scale using the same units for branch length and evolutionary distance used to infer the phylogenetic tree. Evolutionary distance units are in number of base substitutions per site.

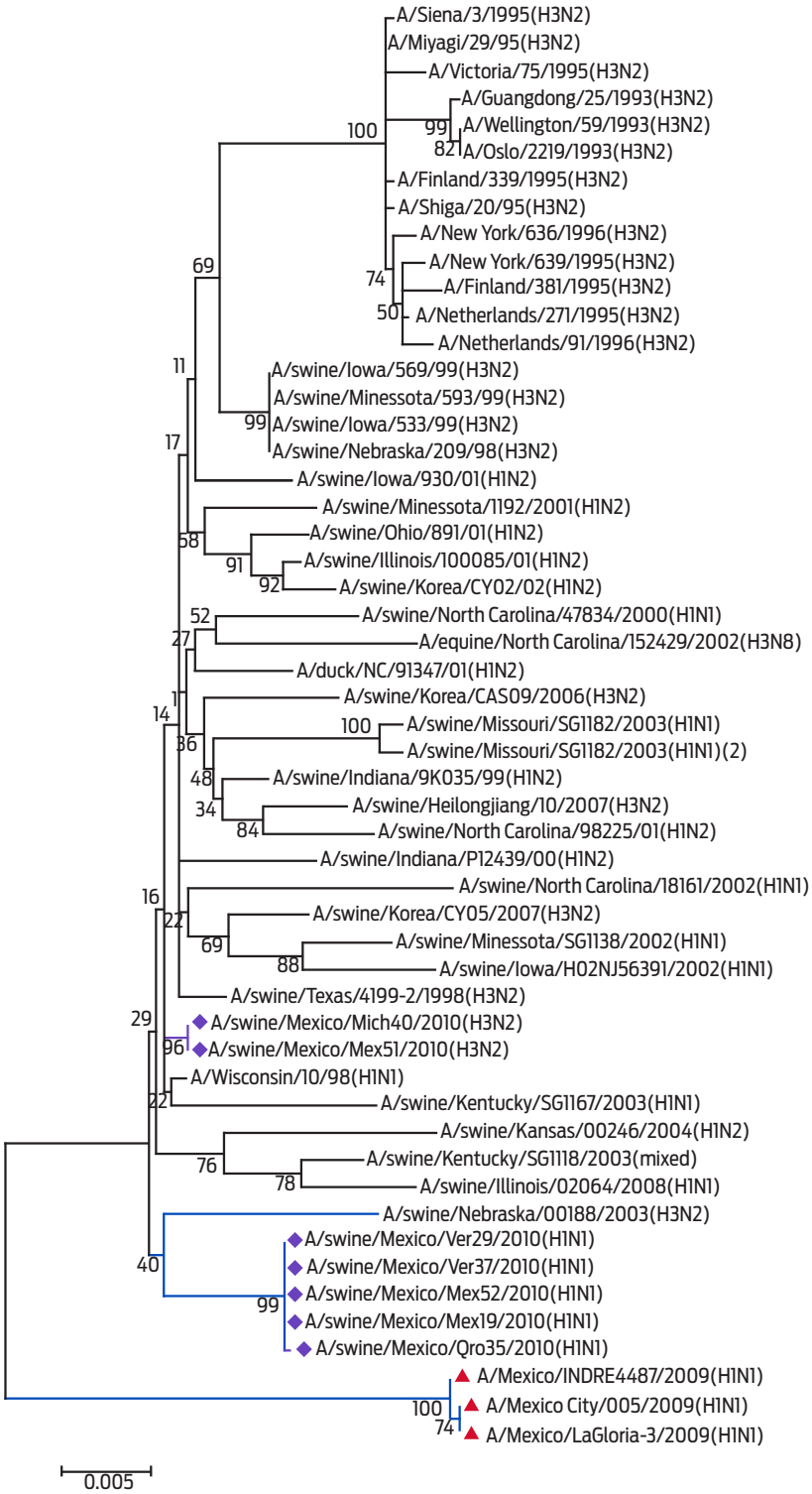

Figure 2. Molecular phylogenetic analysis by the Maximum Likelihood (ML) method using the HKY + G substitution model. Phylogenetic tree of the PB1 gene of the seven viral isolates (violet diamonds) and the pandemic virus (red triangles) based on nucleotide sequences and other sequences in GenBank. The tree is drawn to scale using the same units for branch length and evolutionary distance used to infer the phylogenetic tree. Evolutionary distance units are in number of base substitutions per site.

Table 3. Amino acid residues of the PB2, PB1, and PA genes in seven virus isolates compared to other viral PB2, PB1, and PA sequences available in GenBank.

Viruses	Internal Genes																						
	PB2								PB1							PA							
	292	386	395	434	451	475	637	731	214	368	464	469	565	574	744	254	259	262	336	356	403	459	551	592
A/swine/Mexico/ Mex19/2010(H1N1)	I	V	V	L	T	M	A	I	R	V	N	A	D	Y	I	S	P	K	Q	K	L	I	K	V
A/swine/Mexico/ Ver29/2010(H1N1)	M	A	A	F	I	L	T	I	R	V	N	A	D	Y	I	S	P	K	Q	K	L	I	K	V
A/swine/Mexico/ Qro35/2010(H1N1)	M	A	A	F	I	L	T	I	R	V	N	A	D	Y	I	N	S	R	L	R	L	V	R	V
A/swine/Mexico/ Ver37/2010(H1N1)	M	A	A	F	I	L	T	I	R	V	N	A	D	Y	I	S	P	K	Q	K	L	I	K	V
A/swine/Mexico/ Mich40/2010(H3N2)	I	V	V	L	T	M	A	V	K	I	D	T	N	F	M	S	P	K	L	K	I	I	R	I
A/swine/Mexico/ Mex51/2010(H3N2)	I	V	V	L	T	M	A	V	K	I	D	T	N	F	M	S	P	K	L	K	I	I	R	I
A/swine/Mexico/ Mex52/2010(H1N1)	M	A	A	F	I	L	T	I	R	V	N	A	D	Y	I	S	P	K	Q	K	L	I	K	V
Pandemics	V	V	A	L	I	L	T	V	K	I	D	T	D	F	M	N	P	R	M	R	L	I	R	I
A/swine/ Minnesota/1192/ 2001(H1N2)	I	V	A	L	I	L	T	V	-	-	-	-	-	-	-	S	P	K	L	K	L	I	R	I
A/swine/Wisconsin/ H02AS8/2002(H3N2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	P	K	L	K	L	I	R	V
A/swine/Korea/ JNS06/2004(H3N2)	M	V	A	L	I	L	T	I	-	-	-	-	-	-	-	N	P	K	L	K	L	I	R	I
A/swine/Illinois/ SG1141/2003(H1N1)	M	V	A	L	I	L	T	I	-	-	-	-	-	-	-	N	P	K	L	K	L	I	R	V
A/Malaysia/25920/ 2003(H3N2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	S	R	L	R	L	V	R	I
A/swine/ Nebraska/00188/ 2003(H3N2)	-	-	-	-	-	-	-	-	K	I	D	T	D	F	I	-	-	-	-	-	-	-	-	
Other sequences	I	V	A	L	I	L	T	V	K	I	D	T	D	F	M	S	P	K	L	K	L	I	R	I

Isolates 40 and 51 (H3N2) showed synapomorphic homology and were grouped into a cluster with pig and poultry virus genes with a common ancestor. The sequences in this cluster were found to diverge from isolates 19, 29, 37, and 52 (H1N1). The clusters have different virus origins in the American lineage (Fig. 3).

Six amino acid changes were detected in this protein, but only in the pandemic sequences. Thirty-three amino acid changes were identified in the A/swine/Mexico/Qro35/2010 (H1N1) virus, and all of them correspond to human viral sequences. On the other hand, three amino acid changes identified in virus isolate 35 (N254, R262, and R356) matched pandemic and human virus sequences. In addition, isolate 35 showed two amino acid mutations (S259 and V459) that have been found only in the A/Malaysia/25920/2003 (H3N2) virus..

The amino acid variant L403I was only found in isolates 40 and 51, while variant R551K was observed in isolates 19, 29, 35, 37, and 52. Viral isolates 19, 29, 37, and 52 have an amino acid change in Q344, which is different from the amino acid at this position in isolates 35, 40 and 51 (L344). The amino acid change at V592 in isolates 19, 29, 35, 37, and 52 was found to be identical to those of A/swine/Illinois/SG1141/2003 (H1N1) and A/swine/Wisconsin/H02AS8/2002 (H3N2) (Table 3).

NP gene

Because the NP gene plays a major role in determining viral species specificity (Scholtissek *et al.,* 1985), the phylogeny of the NP gene (segment 5) was fully characterized. High levels of homology in the BLASTn (98-100%) and BLASTp analysis (98-100%) suggested a close relationship to porcine viruses. The seven isolates analyzed were grouped into a cluster in a monophyletic group. Isolates 40 and 51 (H3N2) were placed in a different evolutionary branch as orthologous genes with ancestral proximity to the A/swine/Minnesota/1192/2001 (H1N2) virus, whereas isolates 19, 29, 35, 37, and 52 (H1N1) were grouped as a separate branch with ancestral proximity to the A/swine/Wisconsin/H02AS8/2002 (H3N2) virus (Fig. 4).

Among the virus isolates analyzed, three unique amino acid changes were identified in NP isolated from the pandemic sequences, and Position T3 of the analyzed viral isolates was found to be the same as in A/swine/Minnesota/1192/2001 (H1N2) and A/swine/Wisconsin/H02AS8/2002 (H3N2) isolates. Virus isolates 40 and 51 showed a unique change in position V425I, as did isolates 19, 29, 35, 37, and 52 at position D101N. Most changes were observed at position 217 (e.g., I217 and T217), although the pandemic sequences showed a V217 variant (Table 4).

M gene

Phylogenetically, M gene sequences are paralogs that diverged from an ancestor not supported by the value of the phylogeny. Isolates 40 and 51 (H3N2) are related to porcine viruses and are closer to the A/swine/Nebraska/209/98 (H3N2) virus, with a bootstrap support value of 98%; by contrast, isolates 19, 29, 37, and 52 (H1N1) are related to the A/swine/Korea/CAS05/2004 (H3N2) virus, with a bootstrap support value of 97 (Fig. 5).

We observed 21 amino acid changes in the M gene in pandemic viruses with respect to the other sequences. The amino acid at position A239T in the M1

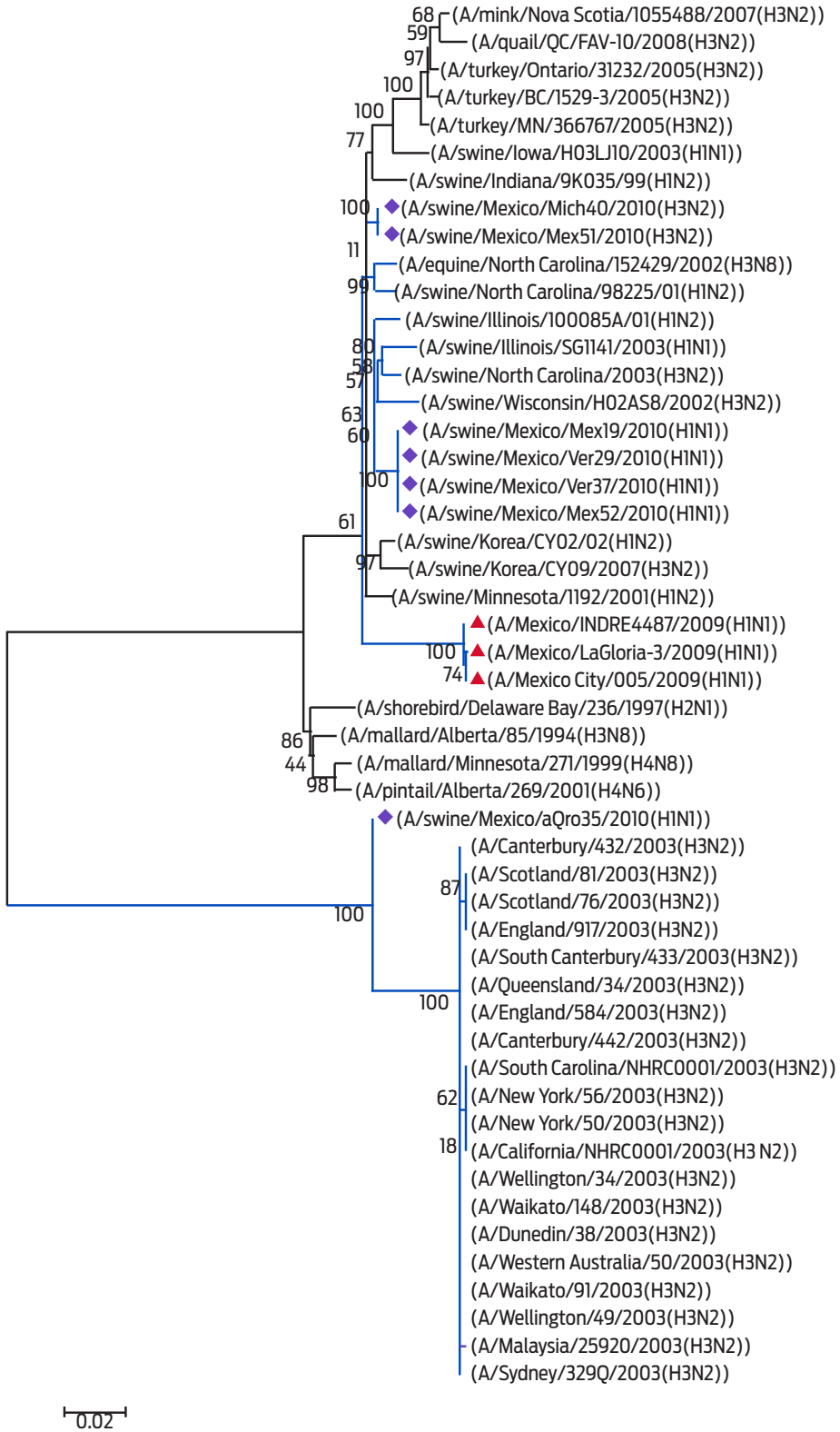

Figure 3. Molecular phylogenetic analysis by the Maximum Likelihood (ML) method using the GTR + G substitution model. Phylogenetic tree for the PA gene of the seven viral isolates (violet diamonds) and the pandemic virus (red triangles) based on nucleotide sequences and other sequences in GenBank. The tree is drawn to scale using the same units for branch length and evolutionary distance used to infer the phylogenetic tree. Evolutionary distance units are in number of base substitutions per site.

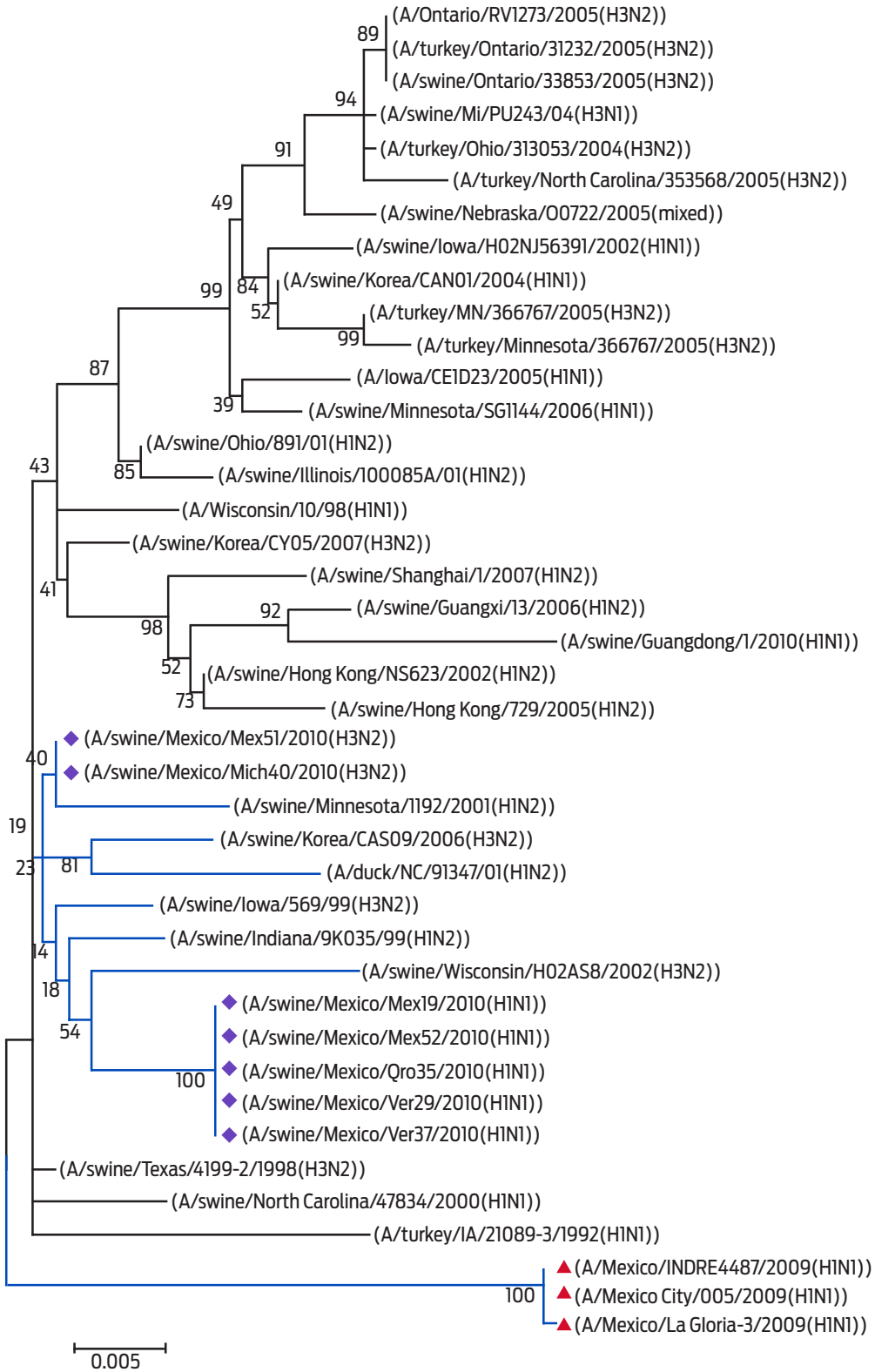

Figure 4. Molecular phylogenetic analysis by the Maximum Likelihood (ML) method using the HKY + G substitution model. Phylogenetic tree for the NP gene of the seven viral isolates (violet diamonds) and the pandemic virus (red triangles) based on nucleotide sequences and other sequences in GenBank. The tree is drawn to scale using the same units for branch length and evolutionary distances used to infer the phylogenetic tree. Evolutionary distance units are number of base substitutions per site.

Table 4. Amino acid residues of the NP, M, and NS genes in seven virus isolates compared to other viral NP, M, and NS sequences available in GeanBank.

VIRUSES	INTERNAL GENES															
	NP				M1		M2		NS							
	3	101	217	425	205	239	54	79	2	27	63	155	157	165	171	194
A/swine/Mexico/ Mex19/2010(H1N1)	T	D	T	I	I	A	C	K	D	L	H	T	I	Y	N	G
A/swine/Mexico/ Ver29/2010(H1N1)	T	D	T	I	I	A	C	K	D	L	H	T	I	Y	N	G
A/swine/Mexico/ Qro35/2010(H1N1)	T	D	T	I	I	A	C	K	D	L	H	T	I	Y	N	G
A/swine/Mexico/ Ver37/2010(H1N1)	T	D	T	I	I	A	C	K	D	L	H	T	I	Y	N	G
A/swine/Mexico/ Mich40/2010(H3N2)	T	N	I	V	V	T	R	E	N	S	Q	A	V	S	D	V
A/swine/Mexico/ Mex51/2010(H3N2)	T	N	I	V	V	T	R	E	N	S	Q	A	V	S	D	V
A/swine/Mexico/ Mex52/2010(H1N1)	T	D	T	I	I	A	C	K	D	L	H	T	I	Y	N	G
Pandemics	S	D	V	V	V	A	R	E	D	L	Q	A	V	S	Y	V
A/swine/ Minnesota/1192/2001(H1N2)	T	D	I	V	-	-	-	-	-	-	-	-	-	-	-	
A/swine/Wisconsin/ H02AS8/2002(H3N2)	T	D	T	V	-	-	-	-	-	-	-	-	-	-	-	
A/swine/Iowa/533/99_(H3N2)	-	-	-	-	V	T	-	-	-	-	-	-	-	-	-	
A/swine/Nebraska/209/98_(H3N2)	-	-	-	-	V	T	-	-	-	-	-	-	-	-	-	
A/swine/Korea/ CAS05/2004(H3N2)	-	-	-	-	V	A	C	K	-	-	-	-	-	-	-	
A/swine/ Texas/4199-2/1998(H3N2)	-	-	-	-	-	-	-	-	D	L	Q	A	V	S	D	V
A/Wisconsin/10/98(H1N1)	-	-	-	-	-	-	-	-	D	L	Q	A	V	S	D	V
Other sequences	S	D	I	V	V	A	R	K	D	L	Q	A	V	S	N	V

subunit matched that in isolates 40 and 51 only, whereas V205I matched isolates 19, 29, 35, 37, and 52. Position C54 in the M2 subunit was identical to that in isolates 19, 29, 35, 37, 52, and A/swine/Korea/CAS05/2004 (H3N2). The amino acid E479 only matched isolates 40, 51, and the pandemic sequences, while K79 matched isolates 19, 29, 35, 37, and 52 (Table 4).

NS gene

A comparison of the NS gene sequences suggested that isolates 19, 29, 35, 37, and 52 (H1N1) belong to a monophyletic group and have a very close synapomorphic relationship to the American viruses A/swine/NewMexico/SG1158/2003 (H3N2), A/swine/Oklahoma/00142/2003 (H3N2), and A/swine/Oklahoma/00142/2003 (H3N2), although they are of different subtypes. On the other hand, isolates 40 and 51 (H3N2) were placed in a separate cluster because they show an evolutionary divergence from the A/swine/Texas/4199-2/1998 (H3N2) swine virus to

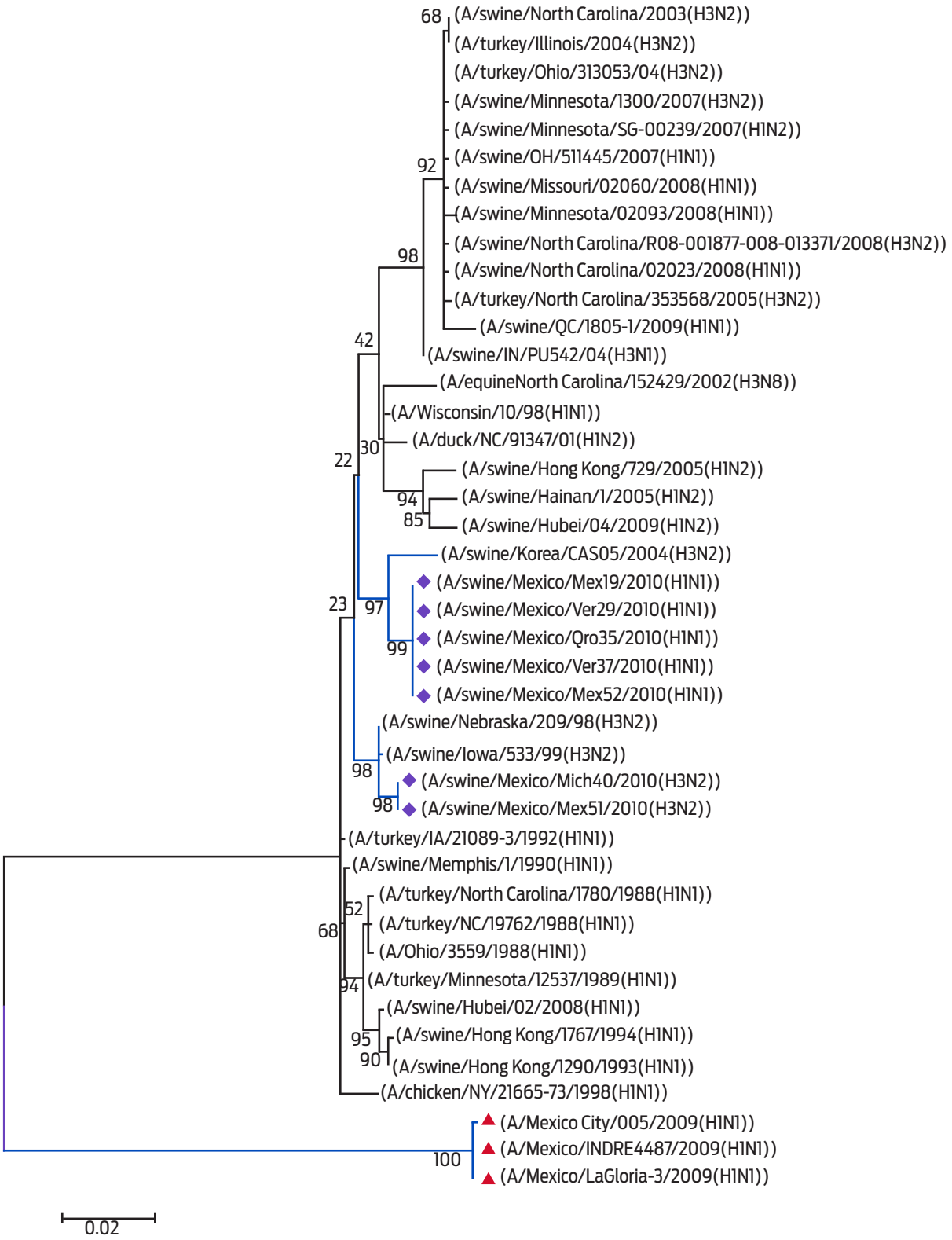

Figure 5. Molecular phylogenetic analysis by the Maximum Likelihood (ML) method using the K2 + G substitution model. Phylogenetic tree for the M gene of the seven viral isolates (violet diamonds) and the pandemic virus (red triangles) based on nucleotide sequences and other sequences in GenBank. The tree is drawn to scale using the same units for branch length and evolutionary distances used to infer the phylogenetic tree. Evolutionary distance units are in number of base substitutions per site.

the A/Wisconsin/10/98 (H1N1) human virus, thereby giving rise to the Mexican H3N2 isolates (Fig. 6).

A total of nine amino acid changes were observed in the NS gene from pandemic viruses compared to the other sequences.

Amino acid variants Q63H, A155T, V157I, S165Y, and V194G were identified in isolates 19, 29, 35, 37, and 52 (H1N1). L27S and D2N were only found in isolates 40 and 51 (H3N2), both of which shared an amino acid change in the position N171D with A/swine/Texas/4199-2/1998 (H3N2) and A/Wisconsin/10/98 (H1N1) isolates (Table 4).

The evolutionary divergence enabled the emergence of a number of influenza viral strains and allowed the progeny virus to establish and maintain itself in different hosts. Several studies have demonstrated that the hemagglutinin gene is the main viral gene involved in host determination. However, internal genes could also play a significant role in host exclusion (Webster *et al.,* 1992).

The lack of systematic surveillance efforts and an appropriate sampling strategy for influenza virus in pig populations in Mexico hampered the timely identification of the H1N1 pandemic in 2009, and the emergence of this virus carrying genes of the Eurasian and North American subtypes was not promptly identified.

In this study, genetic characterization of swine influenza viruses isolated in Mexico during 2009 and 2010, shortly after the pandemic in Mexico, demonstrated a completely divergent phylogenetic relationship with pandemic virus strains. However, the PA gene from A/swine/Mexico/Qro35/2010 (H1N1) showed a genetic relationship with the PA gene from a human seasonal influenza H3N2 virus. This finding suggests reassortment of swine and human viruses, the implications of which are yet unknown.

Rearrangement of genes in the polymerase complex, sometimes including the NP gene, has been involved in virulence attenuation or reduced replicative capacity in specific hosts (Rott *et al.,* 1979). In this study, we demonstrated three amino acid changes in the pandemic virus NP gene, which could be the only differences from the non-pandemic virus strains. Escalera *et al.* (2012) demonstrated the phylogenetic relationship of the NP gene of a swine virus isolate from a farm in Queretaro, Mexico, with two pandemic viruses, A/Mexico/LaGloria-4/2009 (H1N1) and A/Mexico/LaGloria-8/2009 (H1N1), isolated from "ground zero", the place where the disease was first identified. However, our isolates did not contain a nucleoprotein similar to those found in the pandemic viruses. A swine virus isolated in the same state (A/swine/Mexico/Qro35/2010 (H1N1)) as that analyzed in our study is different from the isolate reported by Escalera *et al.* (2012). Those authors identified a D53E amino acid polymorphism in their viral isolate, which suggests that the strain is closer to the pandemic virus. However, our isolate (A/swine/Mexico/Qro35/2010 (H1N1)) did not present such a change, suggesting that human-pig transmission had occurred. Ali *et al.* (2012) identified four mutations, G34A, D53E, I109T, and V313I, in the NP gene of a recombinant between the pandemic and swine H1N2 viruses. Our porcine isolates have the V313F variation with respect to the pandemic sequences. These changes are likely to play a role in viral redistribution and host adaptability, as reported by Chen and Shih (2009) and Pan *et al.* (2009). Both groups identified the amino acid at position 313 as being critical for host adaptation: Y313 for humans, F313 for poultry and swine, and V313 for the pandemic viruses.

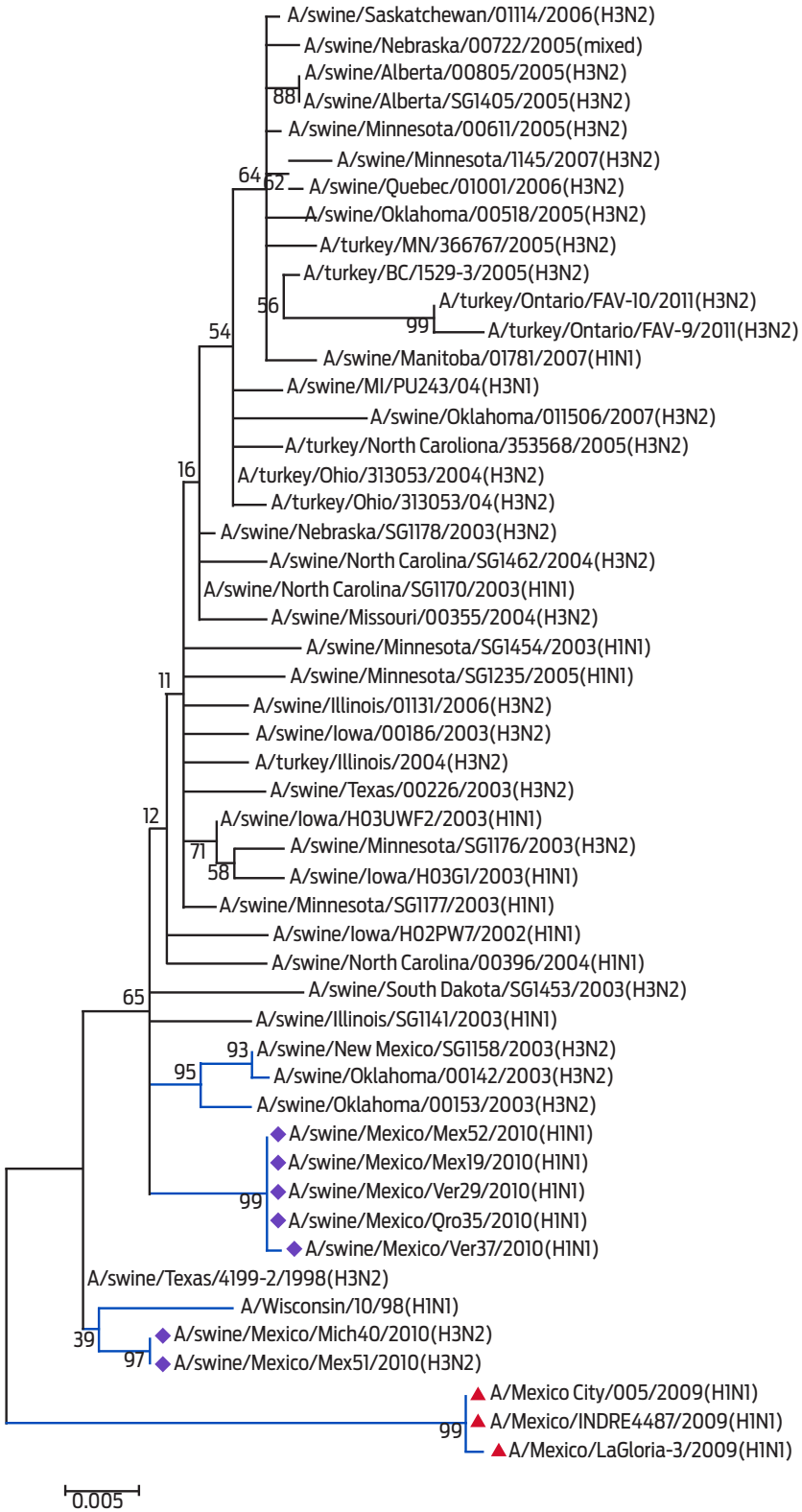

Figure 6. Molecular phylogenetic analysis by the Maximum Likelihood (ML) method using the HKY + G substitution model. Phylogenetic tree for the NS gene of the seven viral isolates (violet diamonds) and the pandemic virus (red triangles) based on nucleotide sequences and other sequences in GenBank. The tree is drawn to scale using the same units for branch length and evolutionary distance used to infer the phylogenetic tree. Evolutionary distance units are in number of base substitutions per site.

The M gene confers species specificity (Scholtissek, 2002). It has been reported that the M gene variants of human and avian viruses can be distinguished by several amino acid substitutions in both Ml and M2 proteins. The S31N mutation of A/H1N1 pdm09 virus was not identified during sequence alignment of the M2 subunit in the M gene in any of the seven porcine isolates reported by the CDC during the 2009 pandemic (CDC, 2009). These viruses contained S31, a mutation known to confer resistance to M2 proton channel inhibitors (amantadine and rimantadine). Only three reference pandemic sequences were retained the N31 mutation (though mutations at positions 26, 27, 30, 31, and 34 were also identified). However, Weingartl *et al.* (2011) observed the same mutation during the genetic characterization of a swine virus isolated from Canadian farms, which confirmed the transmission of the pandemic virus to pigs. The presentation of A/H1N1 pdm09 viral outbreaks in pigs and the direct transmission of the virus from humans to pigs were suggested by Moreno *et al.* (2010) and Pereda *et al.* (2010). The EG21 mutation, which has been suggested as being related to host adaptation, was not detected.

It has been reported that the PB2 gene found in contemporary swine influenza viruses has an avian origin. However, although Mexican swine sequences are phylogenetically unrelated to avian viruses, they carry the avian amino acid variant E627. It appears that E627 is not restricted to the avian PB2 gene and remains in the triple reassortant swine virus lineage. The K627 amino acid change, known to confer adaptation to human cells, was not detected. This was also described by Shu *et al.* (2012) and Ali *et al.* (2012), who reported that the unique mutation V89M confers adaptation to human cells. The sequences reported in this study showed the presence of a valine residue at position 39 (V39).

Our results show that the sequence with the greatest evolutionary distance in number of base substitutions per site per year is the A/swine/Mexico/Qro35/2010 (H1N1) isolate, which possesses thirty-three amino acids identical to those of the human influenza virus.

The amino acid at position 552 is the main regulatory site of the PA protein, capable of adapting it to function in other host cells. According to Mehle *et al.* (2011), the T552S mutation in PA specifically regulates adaptation of the influenza virus to host cells and could lead to affinity to human cells. The authors suggest that an avian virus PA gene mutated and reassorted to enhance the virus infectivity and species tropism. However, with the exception of the A/swine/Mexico/Qro35/2010 (H1N1) sequence that carries the human S552 variant, our pig isolates have sequences similar to those of the pandemic viruses and have maintained their avian traits with respect to the T552 position

Although the internal genes PB1 and NS showed genetic variability among contemporary swine isolates, whether a specific mutation or even a series of mutations contributed to the host adaptation is still unknown. Thus, the role of those amino acid changes in host adaptation needs to be further investigated. Other related pathogenic mutations have been detected in these genes. NS1 is responsible of suppressing antiviral interferon (IFN) induction during viral replication. The D92E mutation in lethal H5N1 strains, which has been associated with increased virulence, was not identified (Seo *et al.*, 2002). However, it was shown that in our pig isolates, residues K317 and I198 in the PB1 protein, which are associated with pathogenicity in mice, were mutated to M317 and K198, respectively (Katz *et al.*, 2000).

Therefore, it is vital to continuously monitor the circulation of new strains of swine flu in Mexico and to estimate the risk of transmission to other species. Human infection with a novel reassortant avian influenza H7N9 virus was identified in China in February 2013 (Gao *et al.,* 2013). It should be noted that the first pandemic influenza outbreak in the 21st century was caused by a novel H1N1 influenza virus of swine origin that emerged in early 2009. This virus is substantially less virulent than the 1918 influenza virus strain but has the potential to acquire amino acid changes in key viral proteins that would increase its pathogenicity. Moreover, it is important to explore host factors that are involved in resistance and susceptibility to influenza virus infection (Watanabe and Kawaoka, 2011).

Conclusions

Our analysis identified changes in the genes of the Mexican swine influenza viruses isolated in 2010 suggesting that these virus strains maintained the entire distribution of the TRIG in PB2, NP, and M genes, an observation not reported in Mexico previously. The most relevant finding in our study is the presence of a PA gene from A/swine/Mexico/Qro35/2010 (H1N1), which is derived from the human H3N2 virus, and the possibility of detecting new virus strains with increased risk of transmission to other species. Therefore, it is important to evaluate whether the observed amino acid changes modify virus pathogenicity and virulence in pigs. As has been demonstrated, the swine population can act as a reservoir of ancestral viral genes of human origin.

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Conflicts of interest

María Elena Trujillo Ortega is Dean of Facultad de Medicina Veterinaria y Zootecnia and José Iván Sánchez Betancourt is Department Head of Departamento de Medicina y Zootecnia de Cerdos, Facultad de Medicina Veterinaria y Zootecnia. The other authors declare that they have no conflicts of interest.

Author Contributions

Víctor Manuel Carrera Aguirre: Redaction and phylogenetic analysis.
María del Carmen Mercado García: Sampling and virus isolation.
María Elena Trujillo Ortega: Manuscript preparation and review.
Susana Elisa Mendoza Elvira: Manuscript preparation and review.
Pavel Isa Haspra: Preparation of genomic libraries.
Luis Felipe Paulin Paz: Sequence assembly.
Carlos Federico Arias Ortiz: Manuscript preparation and sequence analysis.
José Iván Sánchez-Betancourt: Experimental design, redaction, overall responsibility for the project.

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