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Genomic analysis of an atypical Mexican low-pathogenic H5N2 avian influenza virus

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Abstract

We analysed the genome of a low-pathogenic avian H5N2 influenza virus isolated from the faeces of experimentally infected Pekin ducks and Leghorn-type chickens to determine its origin and molecular characteristics. The complete genomic sequence was determined using a Sanger-based genome sequencing method and was subsequently characterized by phylogenetic analysis and genetic comparison. The results of this study showed that 8 genomic segments corresponded to an avian influenza virus that were related with strains isolated in Mexico. Investigation of the haemagglutinin gene revealed the presence of few basic amino acids at the cleavage site and lack of a potential N-glycosylation site at position 11. The gene encoding the PB1 protein lacked PB1-F2 and the basic polymerase gene codes for PA-X. In addition, the basic polymerase gene contained the consensus ribosomal frameshifting motif TCC TTT CGT C, which is required for the expression of the PA-X. Molecular characteristics showed that the virus has features of a low-pathogenic H5 influenza virus with the exception of a potential N-glycosylation site at position 11. The genome information for this particular virus will provide a molecular map for further *in vivo* studies to identify why some influenza viruses can persist in chickens for long periods of time. Such information will be useful in countries such as Mexico, where the virus has been a poultry health problem since 1994 and has the potential to evolve high pathogenicity.

Keywords: Avian influenza; H5N2; Low-pathogenicity; Chicken embryos; Genome.

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Additional information and declarations can be found on page 7

Introduction

The avian influenza virus belongs to the *Orthomyxoviridae* family and the genus Influenzavirus type A. It is known that low-pathogenic avian influenza (LPAI) viruses cause mild to subclinical disease in chickens, but they may become highly pathogenic in chickens after multiple passages in chicken populations (Soda *et al.*, 2011). High-pathogenic avian influenza (HPAI) viruses cause severe disease with high mortality and spread rapidly in short periods of time, resulting in high economical losses. In some cases, these viruses might pose a potential contagious threat to

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mammals, including humans (Causey and Edwards, 2008). LPAI viruses are those that have an intravenous pathogenicity index (IVPI) lower than 1.2 in 6-week-old chickens or less than 75% mortality in 4- to 8-week-old chickens infected intravenously, as well as absence of multiple basic amino acids at the cleavage site of the HA protein (Petrini & Vallat, 2009). Additionally, there are other features related to the pathogenicity of H5 viruses, such as absence of a potential N-glycosylation site in the haemagglutinin protein (Deshpande *et al.*, 1987), amino acid mutations in the PB2 protein of mouse-adapted H5N2 virus (Li *et al.*, 2015), mutations in the PB1-F2 protein in mice and ducks (MacAuley *et al.*, 2010; Marjuki *et al.*, 2010), as well as the presence of a PA-X protein that suppresses viral replication and the host antiviral response in mouse and avian species (Jagger *et al.*, 2012; Hayashi *et al.*, 2015; Hu *et al.*, 2015).

Since December 1994, when the high- and low-pathogenic H5N2 avian influenza viruses were isolated in Mexico, an avian influenza vaccination campaign was established as part of an eradication programme that used inactivated, emulsified, and recombinant pox-avian influenza vaccines. However, only the high-pathogenic avian influenza (HPAI) was eradicated (Villarreal-Chavez and Rivera-Cruz, 2003). Since then, LPAI has been controlled by the officially authorized virus strain A/Ck/México/CPA-232/1994 (H5N2), which is a unique case of long-term massive vaccination of poultry in Mexico. However, major antigenic differences between isolates have been reported through haemogglutination inhibition and virus neutralization tests from viruses isolated between the years of 1993–2002 and 1994–2008, compared to the virus strain A/Ck/México/CPA-232/1994 (H5N2), which suggest that the vaccine does not protect and allows for the circulation of LPAI viruses in specific regions of Mexico (Lee *et al.*, 2004; Escorcia *et al.*, 2010; Armas *et al.*, 2015). Additionally, LPAI virus (H5N2) isolates from chickens in Mexico were genetically closely related to LPAI virus (H5N2) isolates from outbreaks in Taiwan (Chang-Chun *et al.*, 2014) and Central America (Okamatsu *et al.*, 2007).

At present, few isolates of the low-pathogenic virus H5N2 subtype have been reported by the Mexican-American Commission for the Prevention of Foot and Mouth Disease and Other Exotic Animal Diseases (CPA) laboratory, and positive identification of the virus at poultry farms results in massive culling of flocks. So far, the impact of H5N2 virus in Mexico is limited to poultry health, and no high-pathogenicity strain has yet been reported. Most studies of the Mexican LPAIV (H5N2) have been focusing on antigenic drift to justify vaccine development, but other biological or molecular features have not been investigated. During a biological study on the infection with the A/chicken/Mexico/2007(H5N2) influenza virus in specific pathogen free (SPF) Leghorn-type chickens and commercial Pekin ducks, we described that viral replication preferentially occurred in the respiratory tract of chickens and in the digestive tract of ducks and that H5N2 did not cause death in either species but caused mild illness and prolonged shedding of the virus, as determined by RT-qPCR. Furthermore, we discovered that SPF Leghorn-type chickens recovered from illness, which is an interesting trait of this strain (Carranza-Flores *et al.*, 2013). In the present study we characterize the viral genome of A/chicken/Mexico/2007(H5N2) to determine its phylogenetic origin and molecular characteristics associated with pathogenicity to better understand the genetic features related to low-pathogenicity in this particular Mexican strain.

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Materials and Methods

Genome analysis

Viral RNA was extracted from the allantoic fluid (stored at -75°C) using a PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), according to the supplier's specifications. Each of the 8 segments of the viral genome was amplified by RT-PCR with primers following a previously reported protocol (Hoffman *et al.*, 2001) using the Qiagen OneStep RT-PCR Kit (Qiagen, Hilden, Germany). The complete genomic sequence was determined by a Sanger-based genome sequencing method (Sanger *et al.*, 1977) using the BigDye terminator v3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA). The obtained sequences were aligned and edited using MEGA software version 5.05 (Tamura *et al.*, 2011). Each of the 8 segments was compared to sequences available in GenBank using a BLAST algorithm (Altschul *et al.*, 1990). Phylogenetic trees were constructed for each of the 8 genes using a neighbour-joining algorithm using 1000 bootstrap replicates without making assumptions about ancestry or evolutionary history (Tamura *et al.*, 2011). The translation of nucleotide sequences into deduced amino acid sequences was performed by the ExPASy translate tool (Gasteiger, 2003).

Accession Numbers

The nucleotide sequences of the 8 segments obtained in this study were submitted to the GenBank nucleotide sequence database with the following accession numbers: Polymerase basic protein 2 (PB2) [KJ729340]; polymerase basic protein 1 (PB1) [KJ729341]; polymerase acid protein (PA) [KJ729342]; haemagglutinin (HA) [KJ729343]; nucleocapsid protein (NP) [KJ729344]; neuraminidase (NA) [KJ729345]; matrix protein (M) [KJ729346]; and non-structural protein (NS) [KJ729347].

Results and discussion

Genome analysis

The 8 genes of the viral genome encode 11 proteins (PB2, PB1, PA, PA-X, HA, NP, NA, M1, M2, NS1, and NEP) with 759, 757, 716, 252, 564, 493, 449, 252, 97, 230, and 121 amino acids, respectively. Interestingly, the A/chicken/Mexico/2007 (H5N2) virus lacks PB1-F2, but encodes a PA-X protein. Also the PA gene contains the sequence TCC TTT CGT C that allows for a ribosomal frameshift leading to expression of PA-X. In addition, the A/chicken/Mexico/2007 (H5N2) viral genome encodes few basic amino acids at the cleavage site of the HA protein (V-P-Q-R-E-X-R | G-L). Based on the genomic sequence, the HA protein of this virus has 5 potential N-glycosylation sites near the cleavage site at amino acids 27, 39, 179, 302 and 555. Additionally, the HA1 receptor binding site of this H5N2 virus has the amino acid residues Q226 and G228. The neuraminidase has a deletion of 20 amino acids in the stalk region.

A multiple sequence alignment analysis of each genomic segment of the A/chicken/Mexico/2007 (H5N2) viral genome showed that 7 genes have the closest relationship (99% similarity) with the A/chicken/Veracruz/1433-1/2006 (H5N2) virus. The HA gene shared 99% similarity to the HA gene of A/chicken/

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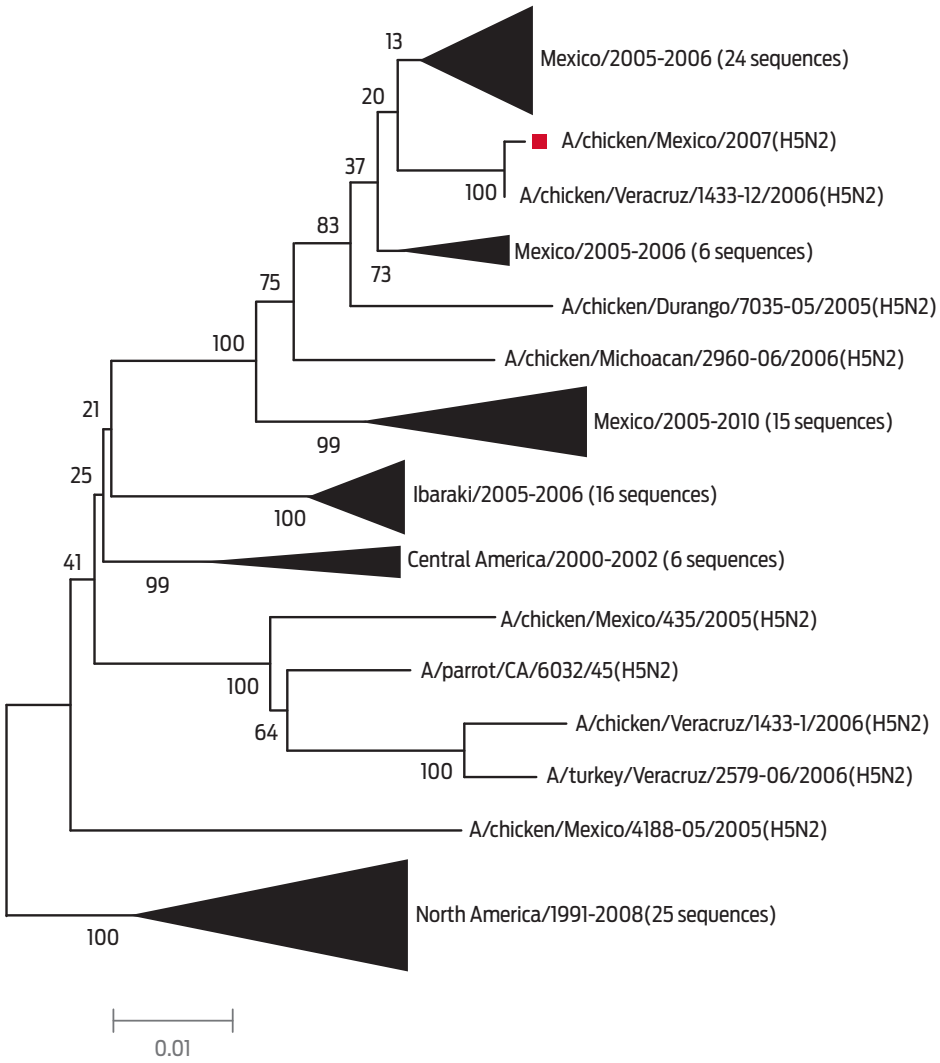

Figure 1. Phylogenetic tree of the HA gene of A/chicken/Mexico/2007 (H5N2) Influenza A virus [■]. The phylogenetic tree was constructed using the neighbour-joining method. The percentages of replicate trees in which the associated taxa clustered together in a bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. The analysis involved 101 chicken nucleotide sequences. Evolutionary analyses were conducted in MEGA 5.05. A larger triangle size represents a larger number of nucleotide sequences with genetic relationship.

Veracruz/1433-12/2006 (H5N2) and 93% similarity to the HA gene of A/chicken/Veracruz/1433-1/2006 (H5N2) (Figure 1). The multiple sequence alignment analysis of the amino acids from each predicted protein produced similar results. All 8 genes had <98% similarity to virus isolates from other states of Mexico. The availability of several complete sequences of contemporary isolated viruses on the GenBank database allowed for an elaborated phylogenetic analysis of the PB2, PB1, PA, NP, NA, M and NS genes between A/chicken/Mexico/2007 (H5N2) and A/chicken/Veracruz/1433-1/2006 (H5N2) as well as an analysis of the HA genes of A/chicken/Mexico/2007 (H5N2) and A/chicken/Veracruz/1433-12/2006 (H5N2). Representative phylogenetic trees showing relationships between 101 chicken nucleotide sequences for the HA (Figure 1) and NA genes (Figure 2) are shown as 15 and 27 branches, respectively, with some branches representing collapsed groups.

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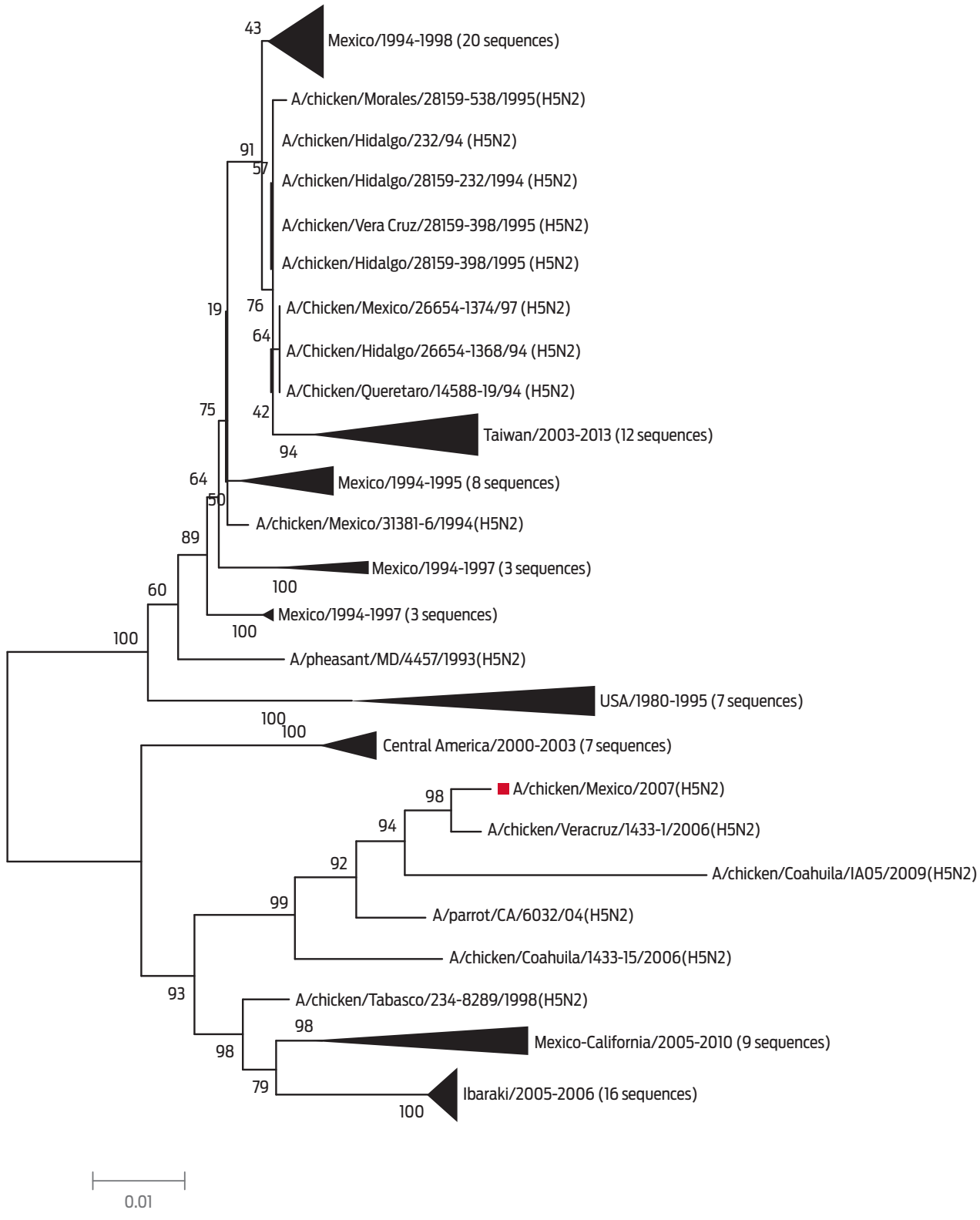

Figure 2. Phylogenetic tree of the NA gene of A/chicken/Mexico/2007 (H5N2) Influenza A virus [■]. The phylogenetic tree was constructed using the neighbour-joining method. The percentages of replicate trees in which the associated taxa clustered together in a bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. The analysis involved 101 chicken nucleotide sequences. Evolutionary analyses were conducted in MEGA 5.05. A larger triangle size represents a larger number of nucleotide sequences with genetic relationship.

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In the figures, larger triangle size represents a larger number of avian nucleotide sequences with genetic similarities.

The genome of the A/chicken/Mexico/2007 (H5N2) virus encodes 11 proteins (Neuman and Kawaoka, 2011). The genome lacks the PB1-F2 gene, which has been associated with increased pathogenicity in avian hosts (Marjuki *et al.*, 2010). The viral genome does encode the PA-X protein that has been shown to represses viral replication and immune response in the mouse and chicken. Together, these findings explain the virus's low-pathogenicity *in vivo* (Jagger *et al.*, 2012; Hayashi *et al.*, 2015; Hu *et al.*, 2015). The genome of the A/chicken/Mexico/2007 virus contains of a conserved ribosomal frameshifting motif that allows for expression of the PA-X gene in low-pathogenic influenza A virus in avian species (Hu *et al.*, 2015). The HA1 receptor binding sequence in this viral genome encode amino acid residues Q226 and G228, which preferentially bind to avian sialic acid receptors in α2-3 linkages (Ha *et al.*, 2001; Zhao *et al.*, 2012). The HA gene of A/chicken/Mexico/2007 has few basic amino acids at the cleavage site of the HA (V-P-Q-R-E-X-R | G-L), which is characteristic of most avian influenza viruses with low pathogenicity (Berhane *et al.*, 2014; Chang-Chun *et al.*, 2014; Wood *et al.*, 1996). However, the lack of a specific potential N-glycosylation site at position 11 near the HA cleavage site could be associated with increased pathogenicity (Deshpande *et al.*, 1987). The neuraminidase exhibits a 20 amino acid deletion at the stalk region, which is an adaptive feature to poultry (Li *et al.*, 2011).

The phylogenetic analyses of A/chicken/Mexico/2007 (H5N2) show that this strain could have originated in 2006 from the Gulf coast state of Veracruz. Veracruz is well connected to other states by highways located in the north and central high plateau, where the main region of poultry farms is located. The 8 genes of the A/chicken/Mexico/2007 (H5N2) were related to low-pathogenic avian influenza viruses from chickens in different states of Mexico, indicating no introduction of foreign subtypes. In addition, the A/chicken/Mexico/2007 (H5N2) HA and NA genes exhibit the highest similarity to A/chicken/Veracruz/1433-12/2006 (H5N2).

Conclusions

The molecular characterization of the present virus shows that most of the molecular features could explain the low-pathogenicity observed in Leghorn-type chickens including the presence of a PA-X protein, the lack of a PB1-F2 protein and absence of polybasic amino acids at the HA cleavage site. The lack of a specific N-glycosylation site at position 11 of the HA cleavage site might be the only feature of this viral genome that can be associated with potential virulence. This characterization of the molecular features of this strain will contribute to understanding how LPAI (H5N2) viruses continue to be endemic in Mexico for long periods of time without evolving high-pathogenicity.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Author contributions

Giovanni Steffani Hernández conducted the bioinformatic analysis and received a scholarship from CONACyT (No. 372444); Edith Rojas Anaya and Elizabeth Loza Rubio analyzed the data and wrote the manuscript; Fernando Chávez Maya did de sequencing and analysed the data; and wrote the manuscript; Gary García Espinosa designed the research, conducted the bioinformatic analysis, analysed the data and wrote the paper.

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