

Complete genome sequence of a velogenic Newcastle disease virus isolated in Mexico

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Abstract In Mexico, the number of cases of the highly virulent Newcastle disease virus is increasing. In 2005, an outbreak of Newcastle disease occurred on an egg laying hen farm in the state of Puebla despite vaccination with the LaSota strain. Farmers experienced a major drop in egg production as a consequence of a field challenge virus. In this study, we characterize the virus, APMV1/chicken/Mexico/P05/2005, responsible for the outbreak. The virus is categorized as a velogenic virus with an intracranial pathogenicity index of 1.99 and a chicken embryo mean death time of 36 h. The complete genome length of the virus was sequenced as consisting of 15,192 bp. In addition, phylogenetic analysis classified the virus as a member of the class II, genotype V. The highly pathogenic nature of the virus has been linked to the amino acid sequence at the fusion protein cleavage site, which contains multiple basic amino acids (RRQKR↓F).

Keywords Avian paramyxovirus · Genetic analysis · Newcastle disease virus · Mexico · Velogenic

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Introduction

Newcastle disease is one of the most contagious avian diseases and is subsequently responsible for some of the greatest economic losses in the poultry industry. Newcastle disease is caused by the Newcastle disease virus (NDV), an enveloped RNA virus from the Paramyxoviridae family, genus *Avulavirus* [1]. The NDV genome consists of single-stranded, non-segmented, negative-sense, RNA. The genome contains the following six genes: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin–neuraminidase (HN), and an RNA-dependent RNA polymerase (L) [2].

Viruses are classified into three pathotypes according to their pathogenicity index, based on chicken embryo mortality: (1) lentogenic viruses that cause mild symptoms with no mortality, (2) velogenic viruses which approach 100 % mortality, and (3) mesogenic viruses which exhibit a moderate pathogenicity with mortality rates ranging from 30 to 50 % with pronounced symptoms; the last two are considered virulent [3]. The pathogenicity of NDV strains is determined primarily by the amino acid sequence at the cleavage site of cellular proteases within the fusion protein. A low proportion of basic amino acids in this site is less susceptible to cleavage by proteases and is therefore considered to be lentogenic. On the other hand, a high proportion of basic amino acids are characteristic of velogenic phenotypes [4]. However, there is wide genetic diversity within this serotype, and various methods have been proposed to classify viruses according to their genome features [5–8].

The most recent classification system, proposed by Czeplédi et al. [7], groups viruses into two major classes based on three genome-size categories, containing 15198, 15186, or 15192 nucleotides. Class I includes NDVs with

genomes containing 15,198 nucleotides, while class II comprises two genome sizes, genotypes I, II, III, and IV containing 15,186 nucleotides and genotypes V, VI, VII, and VIII with 15,192 nucleotides.

It is widely accepted that vaccination using any NDV genotype protects against symptoms of the disease and mortality in the case of a viral outbreak. However, Miller et al. [8] showed that vaccinal protection does not prevent the excretion of viruses and subsequent infection when field strains contain large amino acid differences due to changes in the nucleotide sequence compared to vaccinal strains. Protection improves when the vaccinal strains are phylogenetically more closely related to the challenging strains [8].

Previous reports of isolated NDV strains in Mexico have determined that the most common occurrences belong to genotype V [9–11]. However, the strain most commonly used for vaccination in the last 40 years has been the LaSota strain, belonging to genotype II, followed by some genotype I strains. We isolated a NDV strain, APMV1/chicken/Mexico/P05/2005, henceforth called P05, from an outbreak at a hen farm that occurred despite vaccination and resulted in a significant drop in egg production. In this study, we report our findings on the complete genome characterization of this strain isolated in 2005.

Materials and methods

Isolation and propagation of virus

The strain P05 was isolated in May 2005 from a poultry farm located in the state of Puebla, Mexico. On this farm, an outbreak led to a significant drop in egg production in a hen population that had been vaccinated using the LaSota strain. The virus was isolated from internal organs (lung, trachea, and spleen) of a hen exhibiting severe symptoms of the disease. We used the isolation method extensively described by Alexander in 1989 [11]. Briefly, the organs were collected under sterile conditions, minced to 20 % w/v using Tryptose Phosphate Broth (TPB) as a diluent, centrifuged at 1,500 rpm for 15 min, and 100 μ L of the supernatant of each minced organ were inoculated into the allantoic cavity of 9- to 11-day-old specific pathogen-free (SPF) chicken embryos. The embryos were incubated at 37 °C for 3 days; subsequently, a sample of allantoic fluid was extracted and identified by hemagglutination and specific antibody testing.

Characterization of NDV, HA, and HI testing

To identify the NDV-positive fluid samples, a hemagglutination assay with red cells from chicken embryos was

performed. Positive samples were subjected to HI assay with anti-NDV antibodies to insure that hemagglutination resulted from the NDV HN protein by the method described by Alexander et al. in 1987 [12].

Determination of virulence

Pathogenicity of the P05 strain was determined according to the intracranial pathogenicity index (ICPI) and the mean death time (MDT) of the chicken embryos [11].

Isolation of viral RNA

For isolation of P05 viral RNA, we used allantoic fluid from SPF embryos previously (48 h) infected with the virus. For RNA purification, we used the QIAamp Viral RNA kit (Qiagen).

Oligonucleotide design and RT-PCR amplification

Our oligonucleotide design was based on the alignment of full-length sequences of previously reported NDV genomes. The design considered the non-variable regions in

Table 1 Oligonucleotides used in this study

Name	Oligonucleotide sequence 5' → 3'	Align site
NDV1 ^a	ACCAAACAGA GAATCGGTGA	20
P05-2336	TGTCTCCTGGTATTTATTCC	5632
P05-2335	AATCCCTCCATCAGTGATAC	9242
P05-2338	TCTTGTAATGGACGAAACTC	13,433
P05-2337	ACAGCAATAAGAAACGTATC	10,219
P05-2339	TTCTCAAGTGATGAGGAACC	380
P05-2340	AAGAGGGGTGAGTAAAGTGG	4815
NCV15	CCCTACAGATCTGTATCATT	14,016
NCV16 ^a	ACCAAACAAAGATTTGGTGA	15,173
SP05-1013	AAAATGAAGCAGCTCATGC	1016
SP05-1570	AGACAACGACACCGACTGGG	1583
SP05-3030	TCCTCAATCGACCTGGCTGC	3024
SP05-3390	TTGCTTCTTCCCATCTCCTG	3384
SP05-3920	ATCAATAGTGACATTGAGCGC	3908
SP05-4510	GATCCAGAGTCTTACCCG	4505
SP05-6196	CCCTWGATCAGATGAGAGCC	6196
SP05-6900	AGAACAYCTGAATTTATCC	6907
SP05-10750	TGTCAATCATYTGATTGGCC	10,766
SP05-11440	AGGAYAATGAGGCAGAAGAG	11,446
SP05-8585	CCGAGTTTTATCATTCTCTC	8585
SP05-7780	GCCTGGCAAGGGAYACTACC	7768
SP05-12810	GCACAGTCGAGGAGCACTTC	12,794
SP05-12270	TGAGAATCATTGGATATGTG	12,260

^a NCV1 and NCV16 correspond to 5' and 3' terminal of the viral genome

the genome of the virus with special attention to positions where the insertion of nucleotides has been reported in viral evolution studies. Table 1 shows the oligonucleotides used. The complete genome of the P05 virus was amplified in four overlapping segments using SuperScript[®] III One-Step RT-PCR System with Platinum[®] Taq High Fidelity (Invitrogen) to the following concentrations: viral RNA, 200 ng, 20 μ M of each oligonucleotide, 1 μ L of enzyme mixture, and 25 μ L of 2 \times buffer. The amplification conditions of the thermocycler were as follows: a retrotranscription reaction at 50 °C for 30 min, a PCR at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, hybridization at 50–53 °C for 1 min, amplification at 68 °C for 1 min/kb, and a final 10 min cycle.

Sequencing and analysis

The PCR-generated products were sequenced directly in triplicate using the oligonucleotides shown in Table 1. Sequencing was performed by the Taq FS dye terminator cycle fluorescence-based sequencing method in a Perkin Elmer/Applied Biosystems, Model 3730. Analyses of the resulting genome sequences and genome assembly were performed by means of Geneious software (Biomatters Ltd). For the phylogenetic analysis, we compared the P05 genomic sequence with 76 complete and near-complete reference genome sequences (only sequences >15,180 nt), and of the variable region of gene F (nucleotides 47–420) of viral strains from class I and II (genotypes I–IX), available at GenBank. The evolutionary history was inferred by the Maximum Likelihood method based on the Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA5 [13].

Results and discussion

Virus characterization

Pathogenicity testing showed that the isolated P05 virus can be classified as velogenic. The chicken embryo MDT was

36 h and the ICPI was 1.99, placing the virus in the highly infectious class. Furthermore, the virus is considered to have the highest pathogenicity among viruses previously reported in Mexico [9–11]. In addition, the deduced amino acid sequence at the cleavage site in the gene which encodes for the fusion protein confirmed a high proportion of basic amino acids, characteristic of highly virulent viruses [4].

Genome analysis and deduced proteins

We found that the complete genome of NDV P05 has a length of 15,192 bp (GenBank accession number HM117720). Similar to other avian serotype 1 paramyxoviruses (APMV1), it contains six genes: NP, P, M, F, HN, and L. The G + C % of the viral genome is 46.3 % and shows a decrease from NP to L. Characteristics of the six genes and the deduced protein sequences are shown in Table 2.

The 5' and 3' leader sequences consist of 114 and 55 nt, respectively. The nucleotide sequence of the gene start (GS), gene end (GE), and the length of the intergenic sequence (IG) in the genome of the NDV affect the efficiency of transcription of the six genes [14, 15]. In the P05 strain, similar to other NDV's, the GS sequence of the NP–P–M–F and HN is UGCCCAUCUU with the only change shown in the GS of the L gene UGCCCAUCCU (reference). The GE sequence is relevant for efficient termination and the initiation of the downstream genes [15]. The GE sequences in NDVs differ only slightly between themselves; in the P05 strain, the GE sequences of the six genes show the sequence (U/A)A(U/A)UCUUUUUU. In all genes, the GE sequences of the P05 strain are identical to the Largo/71 strain; however, there is an extra U in the GE sequence of NP of Largo/71. The IG length between genes in P05 are 2 in NP–P, 1 in P–M, 1 in M–F, 31 in F–HN, and 47 in HN–L. Owing to the extra U in the GE of Largo/71, the intergenic sequence between NP and P is just one nt, while in P05 it is two nt.

The complete genome of P05 strain is similar to the previously reported Largo/71 strain [16]. Major similarities between the two strains were identified at the NP and L

Table 2 Genomic characteristics and deduced amino acids

Gene	Intergenic sequence	Nucleotides length (nt)	5'UTR	ORF length (nt)	% G + C	3'UTR	Deduced amino acid length (aa)
NP	2	1,752	66	1,470	50.3	216	489
P	1	1,451	83	1,188	52.4	180	395
M	1	1,241	34	1,095	47.8	112	364
F	31	1,792	46	1,662	45.8	84	553
HN	47	2,002	91	1,716	45.3	195	571
L	–	6,703	11	6,615	44.2	77	2204
Genome	–	15,192		–	46.3	–	–

genes with 98.0 and 98.6 % similarity in the nucleotide sequence and 99.0 and 99.1 % in the deduced amino acid sequence, respectively. By comparing both strains, we found that the gene showing the greatest difference encodes for protein M (similarity percentages were 92 in nucleotide sequence and 87.7 in amino acid sequence) (Table 3).

In addition, the deduced amino acid sequence of protein F of the P05 strain had a 96.4 % similarity to the Largo/71 strain. However, it also showed similarity rates (96.6 and 96.2 %, respectively) with the Fontana/72 (genotype VI) and Herts/33 (genotype IV) strains (Table 3).

Comparisons of the deduced amino acid sequences of both antigenic proteins (F and HN) of P05 and other strains representative of the eight genotypes showed the lowest similarity percentages between the P05 and the LaSota strain (similarities P05/LaSota: 89.4 and 88.8 %) (Table 3). This difference is in accordance with the previous data reported by Miller et al. [17] who showed the greatest genetic diversity between genotypes II and V. This difference is a fact that should be taken into consideration in choice of vaccine seed.

On the other hand, it is well known that the sequence at the F protein cleavage site is the major determinant of pathogenicity in NDV although not the only one [4, 18, 19]. The P05 strain had a pattern of polybasic amino acids in positions 112 through 116 of the F protein (RRQKR↓F). This amino acid sequence is identical to Largo/71 and every NDV isolated in the last 15 years in Mexico [9, 10, 20].

Beyond the fusion protein cleavage site, it has been reported that five other genes in the viral genome may have an effect on viral pathogenicity [18, 19, 21, 22]. Analysis of the deduced amino acid sequence revealed that the P05 strain lacked the mutations previously reported in genes P and L that have been implicated in virulence [21, 22]. This suggests the existence of other not yet characterized regions which may contribute to increased virulence in the P05 strain.

Fig. 1 Phylogenetic analysis of 77 complete and partial genome nucleotide sequences (only sequences >15,180 nt) of class I and class II (genotypes I–IX) of representative genomes. The evolutionary history was inferred by the maximum likelihood method based on the Kimura 2-parameter model. The phylogenetic tree is drawn to scale with the highest log-likelihood (−160892.14) with branch lengths measured in the number of substitutions per site (only shows the highest at 0.005). A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4120)). Codon positions included were 1st + 2nd + 3rd + noncoding. evolutionary analyses were conducted in MEGA5 [13]

Phylogenetic analysis

Phylogenetic analysis of 77 complete and partial genome nucleotide sequences of representative viruses belonging to class I and class II (genotypes I–IX) are shown in Fig. 1. The P05 strain is among the genotype V virus clearly more related to Largo/71. Non-significant changes can be seen in the distribution of the virus in the tree when analyzing the partial sequence of gene F (47–420 nt) (Electronic supplementary material 1). These results match the genotypes of strains isolated in commercial avian species in North America in the last 15 years [9, 20, 23].

The full-length genome sequence of the isolated P05 strain showed a 97.4 % similarity to the Largo/71 strain isolated in the state of Florida in the United States during the initial outbreaks in the early 1970s. These outbreaks have affected various border states between Mexico and the USA [16], but seem to have originated from the import of exotic birds South America [24, 25].

In North America, in commercial poultry, the major genotype identified has been genotype V. For this reason, we made a particular analysis of the variable segment (47–420 nt) of the gene that encodes for the Fusion protein with sequences available in Genbank (majority of viruses isolated in Mexico) (Fig. 2). Our results are similar to those

Table 3 Similarity percentages between the P05 strain and representative strains of each genotype

Genotype:	I		II		III		IV		V		VI		VII		VIII		IX		
Strain:	Ulster (AY562991)		LaSota/46 (AY845400)		JS/7/05/Ch (FJ430159)		Herts/33 (AY741404)		Largo/71 (AY562990)		Fontana/72 (AY562988)		ZJ1 (AF431744)		QH4 (FJ751919)		JS/1/02 (FJ436306)		
	% (nt)	(aa)	% (nt)	(aa)	% (nt)	(aa)	% (nt)	(aa)	% (nt)	(aa)	% (nt)	(aa)	% (nt)	(aa)	% (nt)	(aa)	% (nt)	(aa)	
Gene																			
NP	89.8	94.7	86.5	92.9	89.4	94.3	91.8	95.7	98.0	99.0	92.2	96.5	91.5	96.3	92.0	95.3	89.5	93.3	
P	84.8	81.8	83.7	81.8	86.4	84.3	88.0	86.1	96.8	96.2	89.5	87.6	86.8	84.3	87.9	85.1	85.1	81.3	
M	83.1	81.9	81.5	80.5	83.7	81.1	85.0	81.9	92.0	87.7	86.6	86.0	84.7	84.4	85.5	83.3	82.7	81.9	
F	87.2	91.2	85.9	89.4	87.7	92.1	90.2	94.2	96.9	96.4	91.6	96.6	89.0	93.1	90.3	94.6	88.5	92.4	
HN	87.3	90.4	84.3	88.8	88.0	88.8	90.6	90.6	97.3	96.3	92.1	94.2	89.3	92.7	90.6	93.7	87.1	89.5	
L	89.0	95.5	86.9	94.3	89.7	95.1	91.5	96.9	98.6	99.1	92.8	96.9	90.2	95.7	92.0	97.0	89.7	94.9	
Genome	86.8	–	84.4	–	87.4	–	89.6	–	97.4	–	91.1	–	88.2	–	89.9	–	87.2	–	

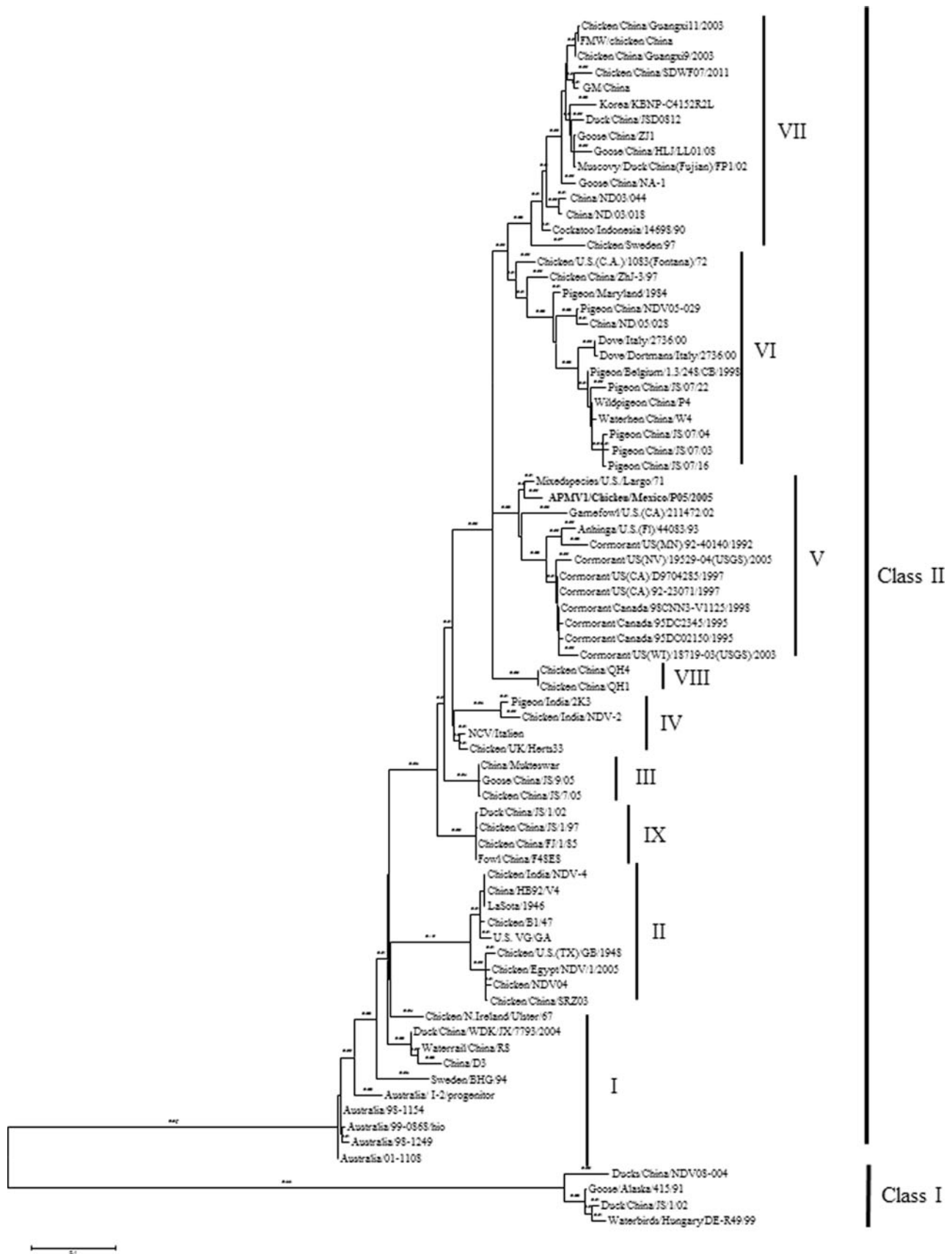
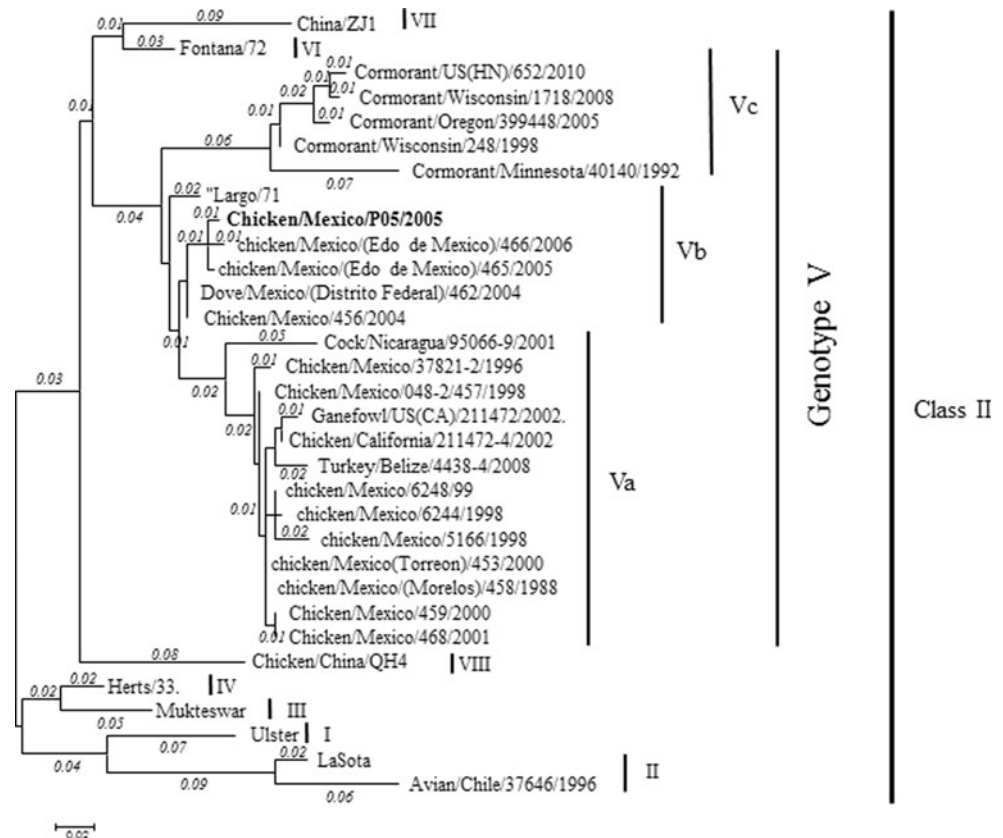


Fig. 2 Phylogenetic analysis of 33 nucleotide sequences of the variable region of gene F (nucleotides 47–420). In the tree, strong emphasis is given to viruses belonging to genotype V. The evolutionary history was inferred by the maximum likelihood method based on the Kimura 2-parameter model. The phylogenetic tree is drawn to scale with the highest log-likelihood (−2012.8672) with branch lengths measured as the number of substitutions per site. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4120)). Evolutionary analyses were conducted in MEGA5 [13]



previously reported by Perozo et al. [9]. Figure 2 shows three lineages of viruses belonging to genotype V. Two of them, have been identified circulating in commercial poultry [9] and a third includes wild birds.

Particular analysis of the virus sequences isolated in Mexico show two lineages with similarities between each group of 92–95 % of the virus circulating in Mexico. The first group (Va) corresponds to isolates between 1998 and 2002 and includes the virus that caused the outbreak in Torreon in 2000. This lineage includes the chicken/Mexico(Morelos)/458/1988 virus and the Gamefowl/U.S.(CA)/211472-4/2002 strain, suggesting that the same variety is circulating in both countries and may have been circulating in Mexico for over 10 years.

The second group (Vb) corresponds to isolates made between 2004 and 2006 with similarities between them greater than 98 %. This group contains the P05 strain and is more closely related to the Largo/71 strain which is representative of the outbreak in North America in the early 1970s.

With these two lineages, the P05 strain shows high similarity (98.5–99.5 %) among strains isolated in Mexico between 2004 and 2006. However, the percentage of similarity of the P05 strain diminishes when compared to strains isolated in Mexico between 1998 and 2001 (93–94 %) [9].

With the information available on commercial chickens, we identified two evolutionary lineages of genotype V in

recent years in North America. The Va lineage which includes the Gamefowl/U.S.(CA)/211472-4/2002 strain and the Vb lineage represented by P05. Probably, both lineages descended from the strain that caused the outbreak in the 1970s. The percentage of similarity between Gamefowl/U.S.(CA)/211472-4/2002 and Largo/71 is 94 %, while the percentage of similarity between P05 and Largo/71 is 97.4 %. This means that the Vb lineage accumulated fewer changes in relation to Largo/71, and the Va lineage has a higher mutation rate in relation to the same strain. These data explain the difference between these two recent strains.

There is little information about NDV isolates in North America from commercial poultry so it is unknown whether the Vb lineage continues to circulate. However, a sequence of the F gene from a virus isolated in 2008 in Belize (Turkey/Belize/4438-4/2008, Genbank access: JN942045) with high similarity to the strain isolated from the Torreon outbreak of 2000 (99 %), suggests that the Va lineage still continues to circulate.

In addition, the phylogenetic tree identifies a third lineage (Vc) which houses the wild virus isolated in the USA from cormorants and is poorly correlated with lineages Va and Vb with differences greater than 9 %. So far, no evidence has been reported that the wild bird strains are circulating in commercial poultry.

Currently, a permanent vaccination program for Newcastle disease is in effect in Mexico. During the last 40 years, vaccination of commercial birds has been carried out using the LaSota strain, categorized as genotype II. It is well known that all NDVs belong to a single serotype so there is protection against mortality in case of a highly virulent challenge. Furthermore, it has been reported that other APMV may confer cross or partial protection against highly virulent NDVs [26]. Despite the protection in mortality that may be offered by vaccination, some reports suggest that vaccination using a virus that resembles the challenge virus contributes to diminished viral shedding [8]. It is necessary, therefore, to know which virus phylogenies circulate in commercial poultry and try to design vaccines closely related to the field virus.

We isolated the P05 strain from a farm with hens vaccinated against Newcastle disease using the LaSota strain. Since the first report of an outbreak of ND in Mexico in 1946, numerous outbreaks have been well documented at the veterinary level, but little is known about the evolution of viral genomes. To our knowledge, this study is the first to provide a complete genome sequence of a viral strain of Newcastle disease isolated in Mexico. Data collected from this study should contribute considerably to the study of the genomic evolution of NDV in Latin America.

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