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Complete genome analysis of velogenic Newcastle disease virus reference strain "Chimalhuacan": evolution of viral lineages in Mexico

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Abstract Newcastle disease virus with velogenic characteristics circulates in the poultry industry in Mexico and various other American countries. In Mexico, vaccine efficacy testing to obtain commercial registration is reliant on a challenge with a velogenic strain known colloquially as Chimalhuacan due to the site where it was isolated. In this paper, we performed a full genome sequencing of the Chimalhuacan strain. The strain belongs to Class II of APMV, particularly genotype V. The viral RNA genome is 15,192 nt in size and contains six genes: 3′ NP-P-M-F-HN-L 5′. The 3′ leader sequence is 55 nt in size and the 5′ trailer sequence 113 nt. The deduced amino acid sequence confirms a velogenic genotype with four basic amino acids at the cleavage site: ^{112}RRQKR$^{\downarrow}$F^{117}. In addition, evolutionary relatedness based on the gene sequence of the fusion protein indicates that this strain is the ancestor of the strains currently circulating in Mexico.

Keywords Chimalhuacan · Newcastle disease virus · Genotype V · Avian diseases · Velogenic

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Introduction

Newcastle disease is one of the most important causes of economic losses in the poultry industry. The disease is caused by an Avian Paramyxovirus type 1 (APMV-1) belonging to the genus *Avulavirus* also known as Newcastle disease virus (NDV) [1]. Depending on its virulence, NDV can cause severe clinical symptoms of the disease with high mortality rates (velogenic); moderate mortality but with manifestations of the effects of the disease (mesogenic virus), and may also present mild symptoms of the disease or be asymptomatic without animal mortality (lentogenic) [2].

Various outbreaks have presented in Mexico caused by NDV [3–6]. The first outbreak of highly pathogenic NDV in poultry presented at the end of the 40s, early 50s as poultry farming in America began to be industrialized [3]. The virus causing this first highly pathogenic outbreak was the Mexico/Querétaro/452/1947 strain [4]. The second highly pathogenic outbreak was in the early 70s with eruptions that affected Mexico and the USA [5]. The Last two outbreaks were in 2000 and 2004 by velogenic virus [6].

In the outbreak of the early 70's, the causative strain was isolated in 1973 at a broiler farm and it became known as the Chimalhuacan strain (Antillon and Lucio, personal communication). Some years later, this high virulent strain was characterized by its intracerebral pathogenicity index (ICPI) of 1.89 [6].

Two decades ago the Mexican government established it as a reference strain to challenge vaccinated birds. Since then, the strain has been used to evaluate the effectiveness of commercially available vaccines in Mexico. Similarly, according to Mexican law, all imported NDV vaccines must be evaluated for protection against a challenge by this strain [7].

In spite of its importance as a reference strain for vaccine efficacy tests, the full genome sequence of the Chimalhuacan strain has not yet been reported. In this paper, we present the full genome sequencing of the Chimalhuacan strain, highlighting the main genetic features. In addition, we made an evolutionary study of the strains circulating in North America and establish that this strain is an ancestor of the strains currently circulating in the region.

Materials and methods

Preservation of the virus

In 1998, an aliquot of Chimalhuacan strain was provided to Investigación Aplicada S.A. de C.V. The aliquot delivered by the government was amplified in specific pathogen-free (SPF) chickens and the allantoic fluid harvested. The allantoic fluid was divided into aliquots in 2.0 mL vials at a 1:1 ratio with glycerin and kept frozen in liquid nitrogen until the start of this analysis.

RT-PCR amplification

A 2 mL vial was taken out of the liquid nitrogen tank and used to infect 9-day-old chicken embryos. The allantoic fluid was harvested at 72 h and used for RNA extraction using "QIAamp Viral RNA Mini Kit". One 3 μL aliquot of RNA containing 150 ng of viral RNA was used as a template for RT-PCR amplification with a high fidelity polymerase system to reduce errors (SuperScript® One-Step RT-PCR System). The viral genome was amplified in 12 overlapping segments of approximately 2 kb using previously reported oligonucleotides [8].

Sequencing and bioinformatic analysis

Sequencing was performed directly on the RT-PCR products using previously reported oligonucleotides in triplicate [8]. Sequencing was performed by the Taq FS dye terminator cycle fluorescence-based sequencing method in a Perkin Elmer/Applied Biosystems, Model 3730. Assembly of the full genome sequence of the Chimalhuacan strain was done using the Geneious (Biomatters Ltd) program. Phylogenetic analysis was done using the full sequence of the F protein from strains of NDV available from GenBank. The evolutionary history was inferred by the Maximum Likelihood method based on the Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA5 [9].

Results and discussion

Assembly of the sequences reveals that the genome of the Chimalhuacan strain is 15,192 nt in size (GenBank accession number KJ577136) with a GC percentage of 46.4 % and shows a decrease from NP to L. Like other

Table 1 Characteristics of genes present in the genome of the Chimalhuacan strain

Gene	nt (length)	nt 5′ UTR	ORF length (nt)	GC (%)	nt 3′ UTR	Deduced amino acid (length)	Start codon	End codon
NP	1,753	55	1,470	50.5	217	489	AUG	UGA
P	1,451	83	1,188	52.7	180	395	AUG	UGA
M	1,241	34	1,095	48.1	112	364	AUG	UAG
F	1,792	46	1,662	45.8	84	553	AUG	UGA
HN	2,002	91	1,716	45.3	195	571	AUG	UAA
L	6,703	11	6,615	44.4	77	2,204	AUG	UAA

Table 2 Nucleotide sequences in the gene end, gene start, and intergenic sequence regions for each gene

Gene start	Gene	Gene end	Intergenis sequence
^{56}UGCCCAUCUU	NP	UAAUCUUUUUUU^{1809}	T
^{1810}UGCCCAUCUU	P	UAAUCUUUUUU^{3260}	T
^{3261}UGCCCAUCUU	M	UAAUCUUUUUU^{45903}	C
^{4504}UGCCCAUCUU	F	AAUUCUUUUUU^{6296}	GUAUUGCUCUUUGGUCA CUUAUAUCCAGUAG
^{6327}UGCCCAUCUU	HN	AAAUCUUUUUU^{8329}	GCUAUCUUCUGUAAGUU GUUUUUUCUUAUAUCCC ACCACCUUUCAUA
^{8376}UGCCCAUCCU	L	UAAUCUUUUUU^{15079}	

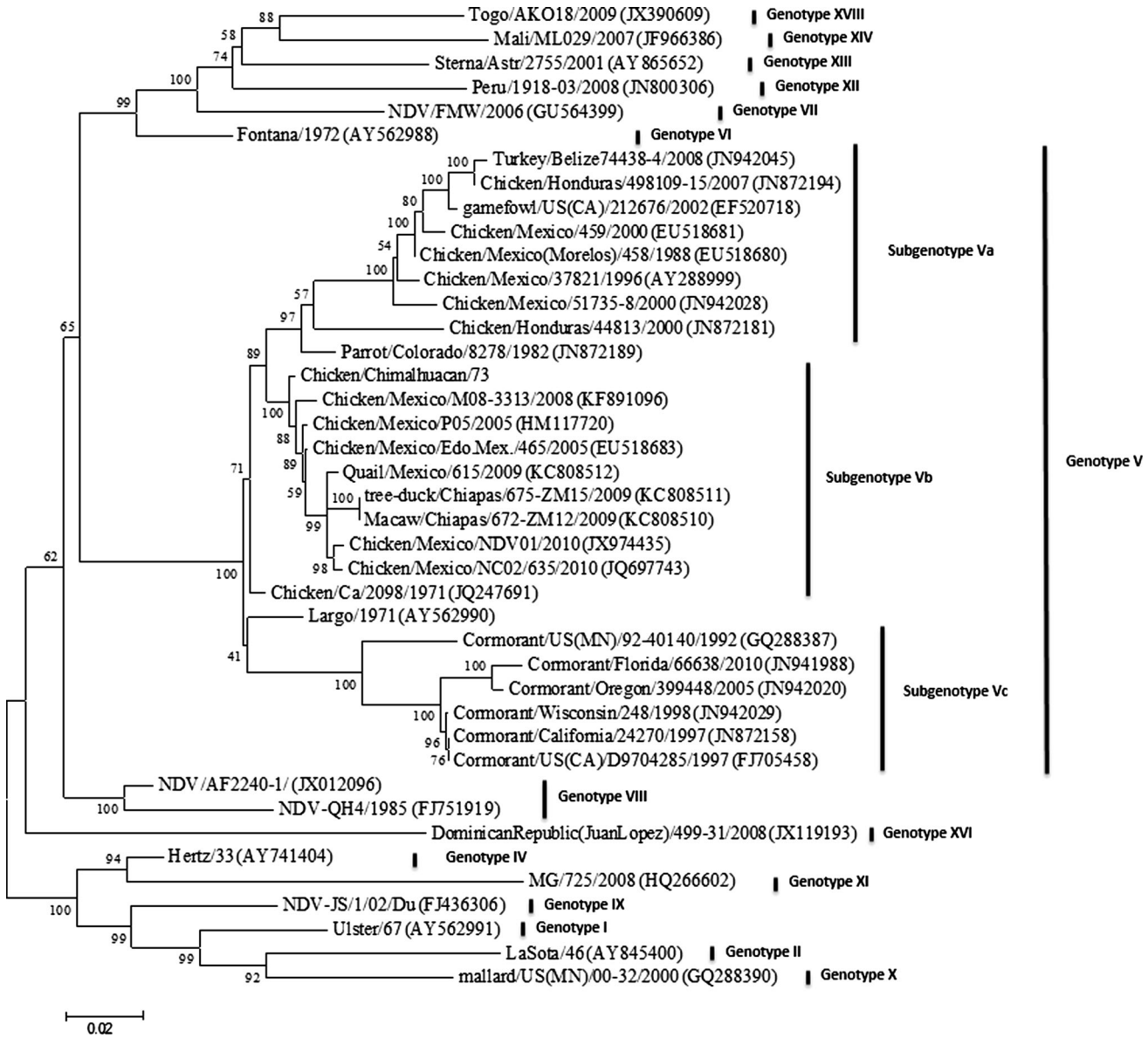

Fig. 1 Molecular Phylogenetic analysis based on the complete F gene sequence of strains of NDV. The viral sequences belong to genotype V, and some representative strains of other genotypes are included as reference. The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model [1]. The tree with the highest log likelihood (−11937.0950) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Evolutionary analyses were conducted in MEGA5 [9]

Newcastle disease viruses it contains 6 genes: Nucleoprotein (NP), Phosphoprotein (P), Matrix protein (M), Fusion Protein (F) Hemagglutinin–Neuraminidase (HN), and Large Polymerase Protein (L). The characteristics of each of the genes in the Chimalhuacan strain genome are summarized in Table 1.

The leader sequence of the 3′ NCR is 55 nt in size followed by the gene start (GS) sequence of the NP gene. A comparison of the leader sequence of the Chimalhuacan strain with other genotype V strains shows little difference between them. Meanwhile, the trailer sequence of the 5′ NCR is 113 nt in size. The GS and gene end (GE) and the intergenic sequence (IG) regions are summarized in Table 2.

The comparison of the nucleotide sequence of the full genome of the Chimalhuacan strain shows similarity percentages of 98.8 % with the Mexico/P05/2005 strain [8], 98.4 % Chicken/Ca/2098/1971, 98.2 % with the Largo/1971 [5] strain, and 98.0 % with the Mexico/NDV01/2010 strain (Online Resource 1). The major differences between the Mexico/P05/2005 strain and the Chimalhuacan strain are in gene M; this is due to considerable changes that present in the P05 strain [8].

Regarding the cleavage site of the Fusion protein (the major determinant of virulence), the deduced amino acid sequence has a RRQKR↓F (aa 112–117) pattern with four basic aa characteristics of highly pathogenic viruses [10]. In addition to the F protein cleavage site, other sites related to virulence identified in the P and L genes were not identified in the Chimalhuacan strain [11].

The evolutionary relatedness analysis showed that Chimalhuacan strain among the Avian Paramyxovirus Class II strains, particularly genotype V, according to the classification criteria based on the of F gene [12].

An evolutionary analysis of strains belonging to genotype V isolated in North America confirms the existence of 3 subgenotypes [8, 13]. Subgenotype Va corresponds to strains isolated before 2002 (also mentioned by some authors as early NDV strains [6]), in which velogenic strains of the "La Laguna" outbreak of 2000 are found. Subsequently, strains of this lineage have been isolated in neighboring countries to the south of Mexico. Subgenotype Vb contains NDVs isolated in 2004 and later; all these velogenic strains present neurotropic and viscerotropic characteristics [8]. The main phenotypic difference between these two lineages is that the strains of subgenotype Vb are more virulent than the strains of subgenotype Va as they have higher ICPI [3, 6]. Subgenotype Vc contains strains isolated exclusively from wild birds (Fig. 1) [13].

The evolutionary history of the viral strains based on the F gene of the NDV showed strains mostly belonging to genotype V, as well as other strains representative of other genotypes. In genotype V, four strains of NDV isolated between 1971 and 1982 stand out: Largo/1971, Chicken/Ca/2098/1971, Chimalhuacan/1973, and Parrot/Colorado/8278/1982. The tree indicates that some of these historic strains are ancestors of the subgenotypes Va and Vb.

The Chimalhuacan strain is a direct ancestor of the strains isolated in 2004 and later, which is supported by the high bootstrap values. Interestingly, the lineage of this strain has increased the degree of virulence measured by the ICPI, given that the Chimalhuacan strain has an ICPI of 1.89 [14], while strains isolated in 2005 and later have ICPI of 1.94 and up to 1.99 [8, 14]. The fact that closely related strains have begun to reappear since 2004 indicates that the strain was possibly maintained in an unknown reservoir and resurfaced with increased virulence. A similar result has been reported by other authors [4] in cases of resurgence of viruses that have remained hidden in unidentified reservoirs for decades before resurfacing.

Meanwhile, the Parrot/Colorado/8278/1982 strain [15] is a probable ancestor of the subgenotype Va (bootstrap 97); this strain was isolated from a parrot that had been in contact with wild birds imported illegally into the country; however, the origin of the birds is unknown [Dr. Nichole Hines personal communication]. The low bootstrap value of Largo/1971 strain does not allow us to infer whether it is an ancestral strain from wild birds, but it may share a close common ancestor with subgenotype Vc.

In this paper, we have presented the genome of the Chimalhuacan strain and indicated the most relevant genetic characteristics. The Chimalhuacan strain is a highly virulent strain used by the Mexican government in vaccine efficacy testing for commercial registration purposes. This strain belongs to genotype V and appears to be the oldest ancestor of subgenotype Vb.

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