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ORIGINAL ARTICLE

Identification of new genovariants of canine distemper virus in dogs from the State of Mexico by analyzing the nucleocapsid gene

Identificación de nuevas genovariantes del virus del distemper canino mediante el análisis del gen de la nucleocápside en perros del Estado de México

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RESUMEN

A nivel mundial, el distemper canino es una de las más importantes enfermedades virales en los perros, debido a su alta mortalidad y morbilidad. Es causada por el virus del distemper canino, un virus RNA, el cual ha demostrado que posee una elevada diversidad genética. En México no existen datos de epidemiología molecular sobre este virus, pero recientemente se ha registrado la presencia de una variante genética aparentemente exclusiva del país. Para determinar la diversidad genética del virus del distemper canino obtenido en el Estado de México, México, en este trabajo se analizaron muestras de perros que presentaban signos de la enfermedad. Para esto, las muestras obtenidas fueron procesadas por RT-PCR y la secuencia nucleotídica de un fragmento del gen N fue obtenida. El análisis de los datos fue realizado mediante filogenia molecular. Los resultados muestran que las secuencias del gen N pertenecen a siete genovariantes del virus del distemper canino que no habían sido previamente reportadas y se encuentran circulando en el Estado de México.

Key words: canine distemper virus, genetic diversity, N gen, new viral genovariants.

Palabras clave: virus del distemper canino, diversidad genética, gen N, nuevas genovariantes virales.

INTRODUCTION

Canine Distemper (CD) is a worldwide, highly contagious disease in young dogs, is caused by the canine distemper virus (CDV) a member of the family *Paramyxoviridae*, genus *Morbillivirus* (Hall 1995). CDV has an envelope composed of a membrane protein termed M and two glycoproteins, the hemagglutinin termed H and the fusion protein termed F (Mochizuki *et al* 1999); the genomic RNA is tightly bound to the nucleocapsid protein, termed N; the major structural protein. The nucleocapsid is a template required for both replication and transcription and comprises the ribonucleoprotein complex in conjunction with the large virus-specified RNA directed RNA polymerase protein (L) and phosphoprotein (P) (Masuda *et al* 2006). The CDV genome is 15,616 nucleotides long, homologous to the non-segmented negative strand RNA virus, consists of a series of contiguous, non-overlapping genes encoding viral structural proteins, flanked by a putative promoter and/or regulatory sequence elements presumably directing the vital processes of genomic transcription, genomic and antigenomic encapsidation and replication.

Thus the genes reading order is 3' to 5' for the virion structural proteins: N, P, M, F, H and L genes (Sidhu *et al* 1993). The N gene is 1,683 nucleotides long, including an open reading frame (ORF) of 1,569 nucleotides, started at the ATG at position 53 to 55 and extended to a terminal codon TAA at position 1,623 to1,625.

Genetic diversity is commonly based on sequencing the hemagglutinin and the fusion protein genes, but recently the N gene is targeted for these types of studies which demonstrated some degree of genetic diversity among CDV isolates, mainly on the regions codifying for the N'- and C' terminal domains (Keawcharoen *et al* 2005). The N protein plays an important role in the virus assembly, replication, and transcription, and also in the infection persistence (Stettler and Zurbriggen 1995). These are some of the reasons why the N gene is a candidate for studies; also the sequences of this gene allow the differentiation among the strains of CDV (Simon-Martinez *et al* 2008).

We conducted a phylogeny-based molecular analysis to identify CDV isolates that are infecting dogs from the State of Mexico, aiming to contribute to future epidemiologic studies. The results obtained showed that the N gene sequences belong to seven Canine Distemper Virus genovariants never before reported in the State of Mexico neither in other countries.

MATERIAL AND METHODS

Blood samples of 15 dogs showing clinical signs of canine distemper virus disease, were obtained from veterinary

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clinics established in Toluca valley; this municipality is located in the central zone of the State of Mexico. The total RNA extraction was performed from leukocytes obtained from 500 µl of blood. The procedure was made using TRI Reagent (SIGMA- Aldrich, USA) following manufacturer's instructions. RNA was eluted in 15 µl of nuclease free water (Fermentas, USA). A pair of primers previously reported by (Shin *et al* 2004) was used to amplify a 297 bp fragment of the N gene. RT-PCR was performed using the Access Quick RT-PCR Kit (Promega, USA). The amplicons were identified by electrophoresis in 2% agarose gels, stained with ethidium bromide (0.5 µg/ml) visualized on a UV transilluminator. Subsequently, PCR products were purified from the agarose gel, using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). 30 ng of purified PCR product were subjected to sequencing analysis using Big Dye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequences were processed on a sequence analyzer 3100 (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were analyzed on an ABI prism® 3130XL (Applied Biosystems, USA). For the genetic diversity analysis, the sequences of the vaccine strain Onderstepoort (GenBank accession number AF014953) and the prototype pathogenic strain A75/17 (GenBank Accession Number AF164967) were used. Other sequences reported worldwide were used too (figure 2).

The multiple sequence alignment, molecular and phylogenetic analysis were realized using the MEGA 4.0 Software (Tamura *et al* 2007). The phylogenetic distances were calculated by the Kimura's two parameters algorithm. With these distances, a phylogenetic tree was constructed using the Neighbor-Joining method. The robustness of the tree was computed by the bootstrap method using 1000 replicates.

RESULTS

RT-PCR amplicons of the 15 cases used in this study produced a 297 bp fragment. However, due to inconsistencies on the extremes of the obtained sequences, these were edited considering only 223 bp for the phylogenetic analysis (corresponding to nucleotides 537 to 759 of the N gen). Seven genovariants were identified out of the 15 sequences obtained in this study, these were named Edomex-1 to Edomex-7 (table 1).

Comparing the sequences of the obtained genotypes from the State of Mexico with the corresponding sequences of the vaccine Onderstepoort strain, there were 20 nucleotide changes, only 11/20 were constant within the State of Mexico genovariants, and allow the differentiation among the pathogenic strains of the State of Mexico and the vaccine Onderstepoort strain (table 2). When comparing the genotypes reported in the State of Mexico isolates, with the pathogenic A75/17 strain, 16 nucleotide changes were observed, of these changes, only six were constant within the EdoMex genovariants. Therefore, the pathogenic genovariants posses seven conserved changes, which allow differentiate them from the vaccine strain Onderstepoort

Table 1. Data of dogs utilized in this study.
Datos de los perros utilizados en este estudio.

Genovariant	GenBank	Case	Age (*)	Sex (†)	Breed	Vaccine (‡)	Region
		1	60	M	Dachshound	–	Toluca
		2	4	F	Border Collie	+	Metepec
EdoMex1	FJ490185	3	2	F	Basset hound	+	Toluca
		4	18	M	Mongrel	U	Metepec
		5	7	M	Schnauzer	+	Toluca
		6	6	F	Poodle	U	Ecatepec
		6	6	F	Poodle	U	Ecatepec
EdoMex2	FJ490186	7	5	M	Mongrel	–	Toluca
		8	3	M	Mongrel	–	Toluca
EdoMex3	FJ490187	9	4	F	Saint Bernard	–	Metepec
EdoMex4	FJ490188	10	2	M	German Shepherd	U	Toluca
EdoMex5	FJ490189	11	3	F	Scotish Terrier	U	Metepec
		12	2.5	M	Mongrel	–	Toluca
EdoMex6	FJ490190	13	2.5	M	Mongrel	U	Toluca
		14	18	M	Akita	–	Toluca
EdoMex7	FJ490191	15	3M	F	Labrador Retriever	–	Toluca

(*) Age in Months; (†) Sex, M = Male, F = Female; (‡) Vaccine, (+) = Vaccinated, (–) = Not vaccinated, U = Unknown.

Table 2. Differences of nucleotides among CDV Mexico State genovariants and strains of pathogenic prototype and vaccine.
Diferencias de nucleótidos entre las genovariantes del VDC del Estado de México y las cepas prototipo patogénicas y vacunales.

	1	12	18	27	39	46	51	84	87	90	99	105	108	114	117	129	138	144	174	177	192	198	213
Onderstepoort (AF014953)	T	A	T	A	A	A	T	G	T	A	C	A	T	G	G	T	C	G	G	A	A	T	C
A75/17 (AF164967)	.	G	.	T	.	G	.	.	C	.	.	C	.	A	.	.	.	.	A	G	G	C	A
CIESAJSMC(DQ887333)	.	G	.	T	.	G	.	A	.	G	.	C	.	A	C	.	.	T	A	G	.	.	.
Edomex1 (FJ490185)	.	G	.	T	G	G	.	A	.	G	.	C	.	A	C	.	.	T	A	G	.	.	.
Edomex2 (FJ490186)	.	G	.	T	.	G	C	A	.	G	.	C	.	A	C	C	.	T	A	G	.	.	.
Edomex3 (FJ490187)	.	G	.	T	G	G	.	A	.	G	.	C	.	A	C	.	T	T	A	G	.	.	.
Edomex4 (FJ490188)	C	G	C	T	G	G	.	A	.	G	.	C	.	A	C	.	.	T	A	G	.	.	A
Edomex5 (FJ490189)	.	G	.	T	G	G	.	A	.	G	T	C	.	A	C	.	.	T	A	G	.	.	A
Edomex6 (FJ490190)	.	G	.	T	G	G	.	A	.	G	.	C	C	A	C	.	.	T	A	G	.	.	A
Edomex7 (FJ490191)	.	G	.	T	G	G	.	A	.	G	.	C	.	A	C	.	.	T	A	G	.	.	A

(table 2). The deduced amino acid sequences show that all genotypes obtained in the State of Mexico possess no difference or changes among them. The amino acid consensus sequences of the 15 State of Mexico genovariants were 100% similar to 51.72% (15/29) of strains reported in the GenBank until the date of this study. Comparing this consensus sequence with the Onderstepoort strain, only one change Asparagine to an Aspartic Acid was observed (Amino acid 159 in the coding sequence of vaccine strain Onderstepoort AF014953) (figure 1).

In the Neighbor-Joining tree, the creations of two principal clades or groups were observed. In the first group, all the State of Mexico genovariants were grouped. In the second group, the creation of two sub-groups was observed; one was formed by pathogenic strains from other world regions and the other by vaccine strains (figure 2).

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                1         10        20        30        40        50        60        70  74
Onderstepoort   LGWLENKDIVDIEVDNAEQFNILLASILAQIWILLAKAVTAPDTAADSEMRRWIKYTQQRRVVGEFRMNKIWLD
LHDLD06 M1      ..............GD.....................................V.....................
H5N07 R2        ..........Y....D.....................................................
LHLD06 M2       ..........G....D.....................................................
SDWD06 M1       ..............D...............................L.......................
LDL05 F1        .........G.....D.....................................................
LDL05 M1        .D............D.....................................................
LH507 R1        .........A.....D.....................................................
HSN07 R1        ..............D.....................................................I.......
ZD01            ..............D.....................................I.....................
SC01            ..............D................T.....................................
HL              ..............D..............V.....................................
Consensus       ..............D.....................................................
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Figure 1. Comparison of amino acid sequences. Multiple alignments of 74 amino acids of vaccine strain Onderstepoort (AF014953) and 11 pathogenic strains reported in GenBank. LHDL06 M1 (EU715247); HSN07 R2 (EU716318); LHLD06 M2 (EU715248); SDWD06 M1 (EU715245); LDL05 F1 (EU715243); LDL051 M1 (EU715241), LHL507 R1 (EU716321); HSN07 R1 (EU716317); ZD01 (EF445050); SC01 (EF042819); HL (EU489475). Consensus sequence of another 15 strains reported in GenBank and all genovariants reported in this study.

Comparasión de la secuencia de aminoácidos. Alineamiento múltiple de 74 aminoácidos de la cepa vacunal Onderstepoort (AF014953) y 11 cepas patógenas reportadas en el GenBank LHDL06 M1 (EU715247); HSN07 R2 (EU716318); LHLD06 M2 (EU715248); SDWD06 M1 (EU715245); LDL05 F1 (EU715243); LDL051 M1 (EU715241), LHL507 R1 (EU716321); HSN07 R1 (EU716317); ZD01 (EF445050); SC01 (EF042819); HL (EU489475). Secuencia consenso de otras 15 cepas reportadas y todas las genovariantes reportadas en este estudio.

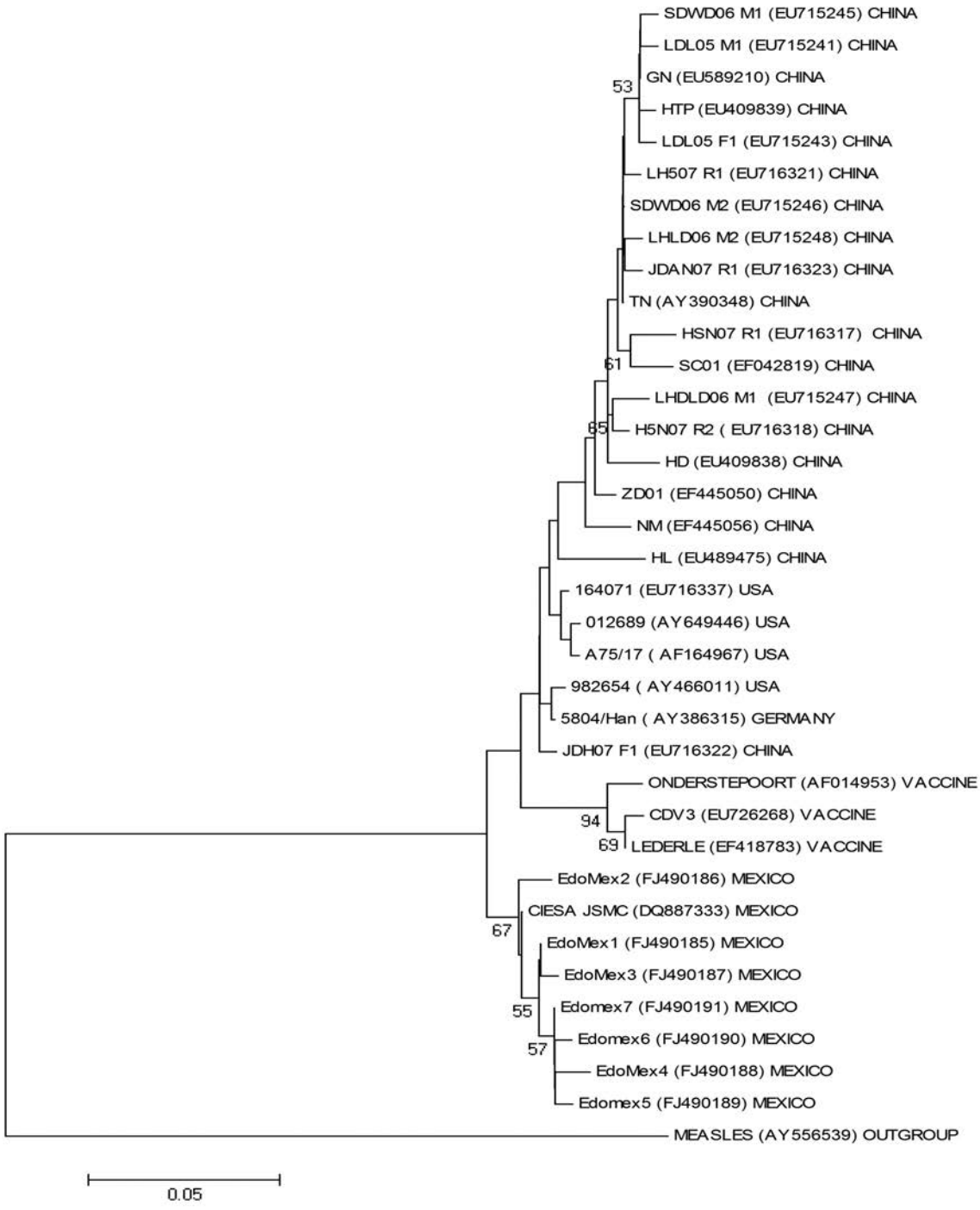

Figure 2. Neighbor-Joining phylogenetic tree was constructed using 1000 bootsrap replicates (the values < 50% are not shown). The phylogenetic distance scale bar indicates the estimated changes per nucleotide.

Árbol filogenético Neighbor-Joining construido usando 1000 repeticiones de bootstrap (no se muestran valores < 50%). La barra de escala de la distancia genética indica los cambios estimados por nucleótido.

DISCUSSION

Canine Distemper is one of the main viral diseases affecting dogs in Mexico. Epidemiological studies of the disease in this country have not been made, probably due to the lack of information about the molecular characteristics of the CDV isolates in the different geographical areas of this country. In order to establish a database of CDV genotypes from Mexico, we did the first CDV genetic typifying based on genetic diversity of the N gene among

the CDV isolates from the State of Mexico. The results obtained showed that different isolates genetically related are circulating in this region and they are exclusive of Mexico; a similar situation has been previously described for other isolates in other countries (Yoshida *et al* 1998, Castilho *et al* 2007). Using this genetic information of the CDV, on the one hand, now we can make the molecular diagnosis of CDV, not only identifying positive cases, but also identifying genovariants that cause the disease, because, at least eight genovariants are circulating in Mexico State; new seven genovariants reported here (EdoMex-1 to EdoMex-7) and the previously reported CIESAJSMC genovariant (Simon-Martinez *et al* 2008). On the other hand, sequencing of the CDV in affected dogs helps to discard cases of CDV produced by vaccine reversion, as in the doubting cases where, recently vaccinated dogs developed the disease, and both clinicians and owners doubt, whether the dog was previously infected with a field virus, or it was a vaccine reversion.

With regards to genetic characteristics, the comparative analysis of the nucleotide sequences among these eight genovariants shows a maximum genetic distance (0.071) between the CIESAJSMC and EdoMex4, and the minimum distance (0.056) between CIESAJSMC and EdoMex1. The sequences of these genovariants, along with the sequences of other isolates reported worldwide were analyzed in a phylogenetic tree. The results show the formation of two main groups, one that includes only the isolates of the State of Mexico, and another formed by two sub-groups. One sub-group includes the pathogenic isolates reported in other countries, and the other sub-group includes the vaccine strains.

Interestingly, all viruses obtained in this study from dogs with a previous history of vaccination belonged to the EdoMex1 genovariant, this genovariant is highly related to the isolate CIESAJSMC obtained from cases of vaccinated dogs (Simon-Martinez *et al* 2008). In other countries, infections of CDV in dogs with history of prior vaccination, involving new variants of the virus have also been observed (Mochizuki *et al* 1999, Keawcharoen *et al* 2005) .

Currently, there are few studies focusing on the correlation of mutations in the N gene with changes in antigenicity and pathogenicity. Although no test correlating the presence of these new genovariants with vaccine failure in dogs was carried out, data provided in this study about the molecular aspect of these new genovariants are important to increase the knowledge of the genome of CDV. For example, all pathogenic strains used in this study have an Aspartic Acid in the amino acid 159, while the vaccine strain has an Asparagine. This molecular characteristic among the pathogenic strains and vaccine strains allows the use of this site for the differentiation between pathogenic and vaccinal isolates.

Another contribution of this work is related to the identification of new sequences of the N gene. This gene and its protein are currently subject to many studies due to its importance on transcription, replication and encapsidation of the RNA genome (Stettler and Zurbriggen 1995). In the same way, the sequencing of N gene is essential due to its use for molecular diagnostic, mainly in RT-PCR (Shin *et al* 1995, Frisk *et al* 1999, Kim *et al* 2001, Shin *et al* 2004) and Real Time RT-PCR (Elia *et al* 2006, Scagliarini *et al* 2007).

SUMMARY

Globally, Canine Distemper Disease is one of the most important viral diseases in dogs due to its high mortality and morbidity. It is caused by an RNA canine paramyxovirus with an elevated genetic diversity. We previously reported the presence of an apparently exclusive genovariant in the State of Mexico, but we do not have data on molecular epidemiology of this virus. In order to determine his genetic diversity in this State, samples collected from dogs showing clinical signs of Distemper Disease were analyzed. The samples were processed by RT-PCR and the nucleotide sequence of an N gene fragment was obtained. The data analysis was performed using molecular phylogeny. The results showed that N gene sequences belong to seven Canine Distemper Virus genovariants that had never been reported before in the State of Mexico.

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