GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Phylogenetic analyses of the hemagglutinin gene of wild-type strains of canine distemper virus in southern Brazil

F.J. Negrão[1], N.R. Gardinali[1], S.A. Headley[1], A.A. Alfieri[1], M.A. Fernandez[2] and A.F. Alfieri[1]

[1]Laboratório de Virologia Animal,
Departamento de Medicina Veterinária Preventiva,
Universidade Estadual de Londrina, Londrina, PR, Brasil
[2]Departamento de Biologia Celular e Genética,
Universidade Estadual de Maringá, Maringá, PR, Brasil

Corresponding author: A.F. Alfieri
E-mail: aalfieri@uel.br

Genet. Mol. Res. 12 (3): 2549-2555 (2013)
Received April 27, 2012
Accepted September 22, 2012
Published March 11, 2013
DOI http://dx.doi.org/10.4238/2013.March.11.2

ABSTRACT. This study examined the phylogenetic relationship of strains of canine distemper virus (CDV) collected from Paraná State, Brazil, based on the hemagglutinin gene. Urine samples were collected from 4 dogs from northern Paraná State that demonstrated clinical manifestations of canine distemper. The participation of CDV was initially confirmed by RT-PCR targeting the nucleocapsid protein, after which the complete hemagglutinin gene was sequenced from each sample. Sequences were deposited in and compared with those already in GenBank. Phylogenetic analyses, using amino acid and nucleotide sequences based on the hemagglutinin gene, demonstrated that these strains of CDV are closely related to those from the Europe 1 lineage of CDV, with marked differences from other recognized geographical clusters of CDV isolates and from the vaccine strains. The strains of CDV from this region of southern Brazil appear to be related to those from Europe 1.

Key words: Canine distemper virus; Phylogeny; Epidemiology; Vaccines

INTRODUCTION

Canine distemper virus (CDV) belongs to the genus *Morbillivirus*, family Paramyxoviridae, and is antigenically related to the following viruses: rinderpest, peste-des-petits-ruminants, measles, and phocine distemper (MacLachlan and Dubovi, 2011). Canine distemper (CD) occurs worldwide, affects a broad range of mammalian host species, and is highly infectious, particularly in young dogs, resulting in elevated morbidity and mortality (Appel, 1987). The classical presentation of CD is characterized by fever, rhinitis, bronchitis, purulent bronchopneumonia, gastroenteritis, and neurological manifestations (Tipold et al., 1992; Greene and Vandevelde, 2012).

The CDV genome is a single-stranded, negative sense RNA that encodes 6 important proteins: the matrix envelope-associated protein; 2 attachment glycoproteins, fusion and hemagglutinin (H); the nucleocapsid protein; and the transcriptase-associated proteins large polymerase and phosphoprotein (MacLachlan and Dubovi, 2011). The H protein is responsible for viral attachment and cell tropism and is one of the most variable *Morbillivirus* proteins that has been frequently used to evaluate genetic diversity between CDV isolates (Haas et al., 1997; Mochizuki et al., 1999; Greene and Vandevelde, 2012). Additionally, nucleotide (nt) and amino acid (aa) variation in the H gene might disrupt important epitopes by affecting neutralization-related sites (Blixenkrone-Möller et al., 1992; Haas et al., 1997; Iwatsuki et al., 2000).

CD in dogs is significantly reduced in countries with extensive use of attenuated CDV vaccines, which have reduced the number of cases of CD since the 1950s. CD continues to affect dogs in Brazil, where the cost associated with treating the systemic effects of the disease is estimated at US$147.5-160.3 million/year (Headley et al., 2012). Although the incidence of CD has increased in both unvaccinated and vaccinated dogs in developed countries (Blixenkrone-Möller et al., 1993; Decaro et al., 2004; Calderon et al., 2007), CD is still endemic in several urban Brazilian cities (Headley et al., 2012). CD outbreaks in domestic dogs and wild animal populations might be caused by the antigenic drift of wild-type strains of CDV, especially that in the H gene (Greene and Vandevelde, 2012), whereas subclinically infected roaming dogs might be a constant source of infection in some urban canine populations in Brazil (Headley et al., 2012). Additionally, low vaccination rates, lapses in booster frequency, the use of less virulent or nonliving vaccines, and the presence of largely unvaccinated, highly susceptible feral or wild canids and other susceptible carnivores might be associated with CD outbreaks (Greene and Vandevelde, 2012).

The characterization of CDV strains in several animal species from different geographic locations has revealed that the antigenic drift of the H gene determines a geographic pattern for CDV lineages. Currently, the major CDV lineages identified worldwide are vaccine strain or America 1 and 2, Asia 1 and 2, Europe 1, Europe wildlife, Arctic-like, and South Africa (Martella et al., 2006; Woma et al., 2010). Recently, distinct strains of CDV were thought to be circulating in some Brazilian cities (Rosa et al., 2012), but these strains might form part of a larger distinct South American clade of CDV (Panzera et al., 2012). Further, restriction fragment length polymorphism analyses of the 721-bp fragment of the H gene have suggested genetic diversity between Brazilian wild-type CDV and vaccine strains (Negrão et al., 2006). This study describes the phylogenetic characteristics of wild-type strains of CDV based on the H gene detected in dogs from northern Paraná, Brazil.

MATERIAL AND METHODS

Samples were collected from 4 dogs with progressive neurological disease at the Veterinary Teaching Hospital, Universidade Estadual de Londrina, PR, Brazil, between 2004 and 2006. These dogs originated from the cities of Londrina (N = 3) and Maringá (N = 1), northern Paraná, and demonstrated at least one of the following neurological manifestations of CD: myoclonus, seizures, and ataxia of the head and limbs. Urine samples from each dog were obtained during routine hospital procedures, and CD was diagnosed using a reverse transcription polymerase chain reaction (RT-PCR) targeting the nucleocapsid gene and amplifying a 287-bp fragment (Frisk et al., 1999). Thereafter, the samples collected were maintained at -20°C for further processing.

CDV RNA was extracted from an aliquot of 300 µL urine based on the silica/guanidinium isothiocyanate method (Boom et al., 1990) to detect the H gene of CDV. To amplify the H gene, we designed 5 primers sets - H1, H2, H3, H4, and H5 (Table 1) - based on the genomic sequences of CDV strains deposited in GenBank. Sequence alignments and primer designs were performed with CLUSTAL/W Multiple Alignment and Gene Runner version 3.05, respectively.

Table 1. Oligonucleotide primers designed from the H gene of the canine distemper virus for reverse transcription polymerase chain reaction sequence analysis.

Primer	Sequence (5'→3')	Position in genome	Sense
H1			
NAH1	CCA ACA GAC ACT CAA GCA	6833-6850	Forward
NAH2	AAC CGT AAC CCA ATC TCA T	7380-7398	Reverse
H2			
NAH3	AGA TTG CTG AAA GAG GAT A	7292-7310	Forward
NAH4	ACA TAC CTT GGC TTT GGA A	7909-7927	Reverse
H3			
NAH5	GTG GAG CTA CTA CTT CAG	7644-7661	Forward
NAH6	TGT CAA CCG CCC ATA AGA T	8290-8308	Reverse
H4			
NAH7	CTG AGA AAC AAG AAG AAC AA	8181-8200	Forward
NAH8	TCA TCC CAC ACA AAA CAT T	8772-8790	Reverse
H5			
NAH9	GTT TAT TAT GTT TAT GAC CC	8681-8700	Forward
NAH10	ATT CTC TCT TTG ATA TTA CG	9168-9187	Reverse

The RT reaction was performed as follows: an initial denaturation at 70°C for 10 min with a mixture of the extracted RNA added to 20 pmol of each forward primer set. This mixture was then placed on ice for 5 min and added to the RT mix containing 1X PCR buffer (30 mM Tris-HCl, pH 8.4, and 75 mM KCl), 3 mM $MgCl_2$, 0.8 mM of each deoxyribonucleotide triphosphate (Invitrogen™ Life Technology, Carlsbad, CA, USA), 60 U Super Script Reverse Transcriptase (Invitrogen™ Life Technology), and ultrapure sterile water to the final volume of 50 µL. The mixture was incubated at 42°C for 30 min and then inactivated at 70°C for 15 min.

Five steps were performed to amplify the complete H gene of CDV. For each step of amplification, cDNA was added to the PCR mix consisting of 1X PCR buffer (30 mM Tris-HCl, pH 8.4, and 75 mM KCl), 3 mM $MgCl_2$, 0.8 mM of each deoxyribonucleotide triphosphate, 20 pmol of each primer pair, 2.5 U Platinum Taq DNA polymerase (Invitrogen™ Life Technology, São Paulo, SP, Brazil), and ultrapure sterile water to the final volume of the

reaction. The following cycle was used: one step of 4 min at 94°C; followed by 40 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C, and a final step of 7 min at 72°C. The amplified products were analyzed via electrophoresis on 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

The amplicons were purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare™, Little Chalfont, UK) and the Gel Band Purification Kit (GE Healthcare) and quantified in a Qubit™ fluorometer using Quant-iT™ double-stranded DNA BR Assay Kit (Invitrogen™ Life Technologies, Eugene, OR, USA). Sequencing was performed in both directions using the forward and reverse primers corresponding to each PCR amplicon. The quality of each sequence obtained was analyzed using the Phred software; the consensus sequence was determined using the CAP3 software (http://asparagin.cenargen.embrapa.br/phph/). The nt identity was verified with sequences deposited in GenBank using the Basic Local Alignment Search Tool software (http://www.ncbi.nlm.nih.gov/BLAST). An nt-deduced aa alignment and phylogenetic tree were created using Molecular Evolutionary Genetics Analysis 4.1; the sequences used during this study are shown in Figure 1.

The sequence identity matrix was determined using BioEdit version 7.0.8.0. A phylogenetic rooted tree was constructed using the neighbor-joining method with Kimura 2-parameter distance, and bootstrap values were determined using 1000 replicates. The gene sequences of the strains identified in this study were deposited in GenBank (CDVBR1, EU098102; CDVBR2, EU098103; CDVBR3, EU098104; CDVBR4, EU098105). The partial sequences of the H gene derived from a recent study carried out in Campinas, SP, Brazil (Rosa et al., 2012), were not located in GenBank and consequently excluded from our study.

RESULTS AND DISCUSSION

The desired amplicons of the H gene of CDV were amplified in all urine samples. A fragment of 2355 bp was obtained in which 1821 bp corresponded to the H gene that coded a 607-aa fragment. Figure 1 shows that the wild-type strains of CDV identified in this study were grouped into one distinct cluster (the southern Brazilian clade), whereas the nt identity between these local strains ranged from 97.1 to 99.3%. Within this clade, the nt-deduced aa sequences of dogs originated from the city of Londrina (GenBank accession Nos. EU098103, EU098104, and EU098105) formed a subgroup that was distinct from that derived from Maringá (EU098102). The Brazilian cluster demonstrated elevated nt identity (97.1-98.5%) with the European cluster. However, a comparatively reduced identity (91.7-92.7%) was observed when these sequences were compared with the vaccine lineage (America 1). The identity differences obtained by comparing nt sequences from the Brazilian strains of CDV with other main clades (Asia 1 and 2, America 2, Europe 1, European wildlife, Arctic-like, and South Africa) ranged from 93.1 to 96.4%.

A lineage is characterized on the level of aa diversity within and between different phylogenetic clusters. Intra-lineage variation has an aa diversity of less than 3.5%, whereas the aa diversity between groups is greater than 4% (Bolt et al., 1997; Martella et al., 2006). In the present study, the aa identity of the CDV strains in southern Brazil varied between 96.5 and 99.0%, showing less than 3.5% aa divergence. The Brazilian cluster presented aa variation between 1.7 and 4% with the Europe 1 lineage. Therefore, although the Brazilian

samples clustered in a separated clade, they should be considered to originate from the Europe 1 lineage. Similar results have been described (Panzera et al., 2012). This combined cluster of CDV strains from Europe and South America might represent the largest known group of CDV strains circulating worldwide. Further, the results demonstrated that the Brazilian cluster might be part of a larger distinct clade of South American strains of CDV in which 2 distant lineages of CDV have been identified (Panzera et al., 2012). Notwithstanding this larger intercontinental feature, the Brazilian strains identified in this study formed a distinct clade of circulating strains of CDV. Additionally, these initial results suggest that the wild-type strains of CDV circulating in these two Brazilian cities might be different.

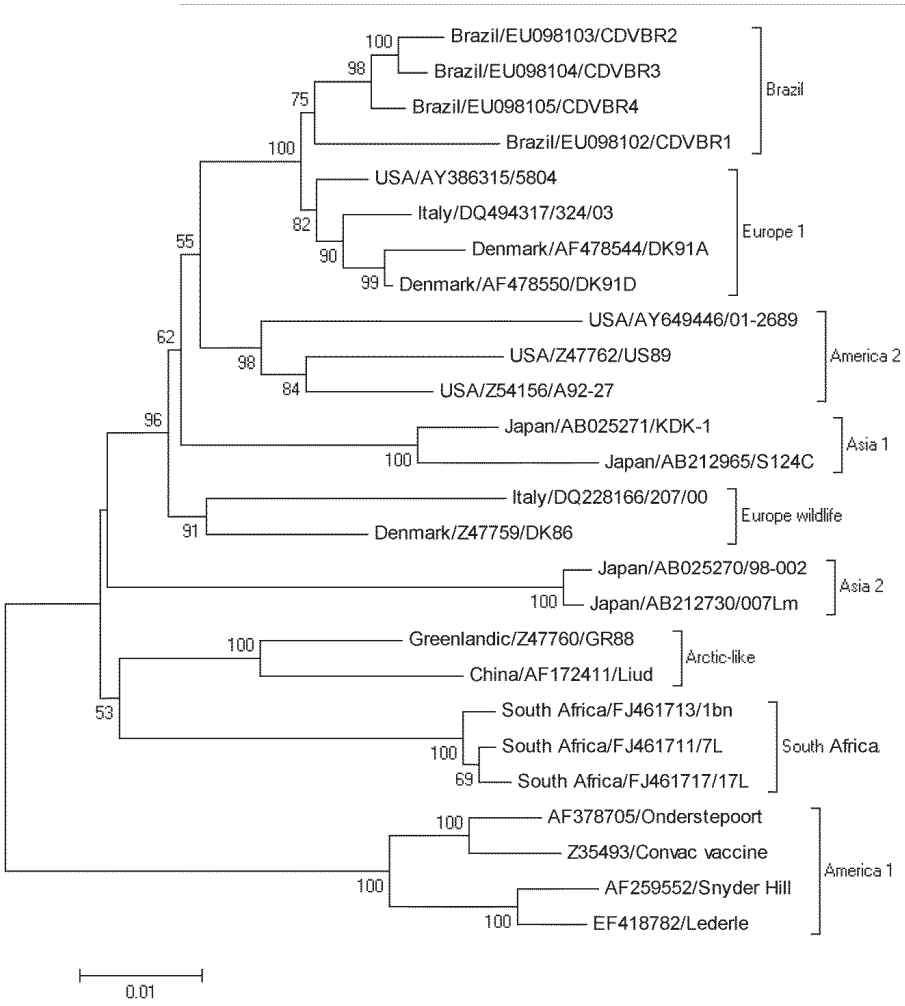

Figure 1. Phylogenetic tree based on the nucleotide sequences of the 1821-bp region within the hemagglutinin gene of the canine distemper virus (CDV) genome. Bootstrap values are indicated when >50% of the 1000 bootstrap replications supported the branch in the consensus tree. The 8 major CDV lineages are indicated.

The southern Brazilian strains of CDV displayed elevated aa variation (7.5-9.1%) compared with that of the vaccine lineage (America 1). Similar results have been described in other studies (Martella et al., 2006; Woma et al., 2010), which have demonstrated that the CDV strains used in vaccines (old strains) are distantly related to novel CDV field strains. Additionally, repeated attenuation of commercially produced vaccines and the concomitant presence of other viral agents within these vaccines might induce genetic drift (Woma et al., 2010) and consequently alter vaccine efficacy. This genetic variation can affect the host immune response, but the real effects of this diversity on the currently used vaccines remain unknown. Consequently, the genetic drift demonstrated by the wild-type Brazilian strains of CDV should be considered a possible cause of outbreaks in local urban vaccinated canine populations. Additionally, most vaccine strains of CDV were isolated in the USA between 1930 and 1950, and these differ genetically from virulent isolates (Greene and Vandevelde, 2012).

Phylogenetic analyses of the H gene of CDV isolated from dogs and other carnivores have demonstrated that wild-type strains of CDV differ by geographic distribution rather than by host species (Harder et al., 1996; Bolt et al., 1997; Carpenter et al., 1998). In this study, the Brazilian wild-type strains of CDV clustered with European strains, indicating that CDV strains in these different geographical regions are likely to have a common origin. However, the territorial extension of Brazil is of continental significance, which can support the possible concomitant circulation of different lineages of CDV. Consequently, detailed and extensive epidemiological surveys are required to determine the circulating lineages of CDV within Brazil and to understand the global ecology of CDV. Nevertheless, the differences in genetic drift observed between vaccines and the recently described isolates worldwide should be considered to understand the effectiveness of currently produced vaccines and the resurgence of CD in dogs and wildlife (Martella et al., 2006). Although the strains described in this study were considered Brazilian strains of CDV (Panzera et al., 2012), we believe that they should be referred to as CDV strains circulating in southern Brazil, or more specifically, in northern Paraná, so that the continental extension of Brazil be taken into account.

In conclusion, we demonstrated that the strains of CDV circulating in southern Brazil are genetically related to European strains but are distantly associated with current vaccine strains. We remain uncertain whether genetic alterations in the Brazilian strains of CDV might be responsible for distemper outbreaks in local vaccinated canine populations. Consequently, the genetic basis of the H gene diversity of CDV in other geographical regions of Brazil must be elucidated to enhance the development of new vaccines.

ACKNOWLEDGMENTS

Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Financiadora de Estudos e Projetos, and Fundação Araucária. S.A. Headley, A.F. Alfieri, and A.A. Alfieri are recipients of Conselho Nacional de Desenvolvimento Científico e Tecnológico fellowships.

REFERENCES

Appel M (1987). Canine Distemper Virus. In: Virus Infection of Carnivores (Appel MJ, ed.). Elsevier Science Publishers B.V., Amsterdam, 133-159.

Blixenkrone-Möller M, Svansson V, Appel M, Krogsrud J, et al. (1992). Antigenic relationships between field isolates of

morbilliviruses from different carnivores. *Arch. Virol.* 123: 279-294.
Blixenkrone-Möller M, Svansson V, Have P, Orvell C, et al. (1993). Studies on manifestations of canine distemper virus infection in an urban dog population. *Vet. Microbiol.* 37: 163-173.
Bolt G, Jensen TD, Gottschalck E, Arctander P, et al. (1997). Genetic diversity of the attachment (H) protein gene of current field isolates of canine distemper virus. *J. Gen. Virol.* 78: 367-372.
Boom R, Sol CJ, Salimans MM, Jansen CL, et al. (1990). Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28: 495-503.
Calderon MG, Remorini P, Periolo O, Iglesias M, et al. (2007). Detection by RT-PCR and genetic characterization of canine distemper virus from vaccinated and non-vaccinated dogs in Argentina. *Vet. Microbiol.* 125: 341-349.
Carpenter MA, Appel MJ, Roelke-Parker ME, Munson L, et al. (1998). Genetic characterization of canine distemper virus in Serengeti carnivores. *Vet. Immunol. Immunopathol.* 65: 259-266.
Decaro N, Camero M, Greco G, Zizzo N, et al. (2004). Canine distemper and related diseases: report of a severe outbreak in a kennel. *New Microbiol.* 27: 177-181.
Frisk AL, König M, Moritz A and Baumgartner W (1999). Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. *J. Clin. Microbiol.* 37: 3634-3643.
Greene CE and Vandevelde M (2012). Canine Distemper. In: Infectious Diseases of the Dog and Cat (Greene CE, ed.). 4th edn. Elsevier, St Louis, 25-42.
Haas L, Martens W, Greiser-Wilke I, Mamaev L, et al. (1997). Analysis of the haemagglutinin gene of current wild-type canine distemper virus isolates from Germany. *Virus Res.* 48: 165-171.
Harder TC, Kenter M, Vos H, Siebelink K, et al. (1996). Canine distemper virus from diseased large felids: biological properties and phylogenetic relationships. *J. Gen. Virol.* 77: 397-405.
Headley SA, Amude AM, Alfieri AF, Bracarense APFRL, et al. (2012). Epidemiological features and the neuropathological manifestations of canine distemper virus-induced infection in Brazil: a review. *Semina*: *Ciênc. Agrárias* 33: 1945-1978.
Iwatsuki K, Tokiyoshi S, Hirayama N, Nakamura K, et al. (2000). Antigenic differences in the H proteins of canine distemper viruses. *Vet. Microbiol.* 71: 281-286.
MacLachlan NJ and Dubovi EJ (2011). Paramyxoviridae. Fenner's Veterinary Virology. 4th edn. Academic Press, San Diego, 299-325.
Martella V, Cirone F, Elia G, Lorusso E, et al. (2006). Heterogeneity within the hemagglutinin genes of canine distemper virus (CDV) strains detected in Italy. *Vet. Microbiol.* 116: 301-309.
Mochizuki M, Hashimoto M, Hagiwara S, Yoshida Y, et al. (1999). Genotypes of canine distemper virus determined by analysis of the hemagglutinin genes of recent isolates from dogs in Japan. *J. Clin. Microbiol.* 37: 2936-2942.
Negrão FJ, Wosiacki SH, Alfieri AA and Alfieri AF (2006). Restriction pattern of a hemagglutinin gene amplified by RT-PCR from vaccine strains and wild-type canine distemper virus. *Arq. Bras. Med. Vet. Zootec.* 58: 1099-1106.
Panzera Y, Calderón MG, Sarute N, Guasco S, et al. (2012). Evidence of 2 co-circulating genetic lineages of *canine distemper virus* in South America. *Virus Res.* 163: 401-404.
Rosa GN, Domingues GH, Santos MMAB, Felippe PAN, et al. (2012). Molecular detection and phylogenetic analysis of the gene H from canine distemper virus isolates circulating at the municipality of Campinas, São Paulo. *Pesq. Vet. Bras.* 32: 72-77.
Tipold A, Vandevelde M and Jaggy A (1992). Neurological manifestations of canine distemper virus infection. *J. An. Pract.* 33: 466-470.
Woma TY, van Vuuren M, Bosman AM, Quan M, et al. (2010). Phylogenetic analysis of the haemagglutinin gene of current wild-type canine distemper viruses from South Africa: lineage Africa. *Vet. Microbiol.* 143: 126-132.