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Short communication

Evidence of two co-circulating genetic lineages of *canine distemper virus* in South America

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ABSTRACT

Canine distemper virus (CDV) is the etiological agent of a multisystemic infection that affects different species of carnivores and is responsible for one of the main diseases suffered by dogs. Recent data have shown a worldwide increase in the incidence of the disease, including in vaccinated dog populations, which necessitates the analysis of circulating strains. The hemagglutinin (H) gene, which encodes the major antigenic viral protein, has been widely used to determine the degree of genetic variability and to associate CDVs in different worldwide circulating lineages. Here, we obtained the sequence of the first full-length H gene of field South American CDV strains and compared it with sequences of worldwide circulating field strains and vaccine viruses. In South America, we detect two co-circulating lineages with different prevalences: the Europe 1 lineage and a new South America 2 lineage. The Europe 1 lineage was the most prevalent in South America, and we suggest renaming it the Europe 1/South America 1 lineage. The South America 2 lineage was found only in Argentina and appears related to wild CDV strains. All South American CDV strains showed high amino-acid divergence from vaccine strains. This genetic variability may be a possible factor leading to the resurgence of distemper cases in vaccinated dog populations.

Canine distemper is one of the most severe infectious diseases in wild and domestic dogs and in many other carnivores. It is distributed worldwide and results in high morbidity and mortality rates (Appel, 1987). The etiologic agent of canine distemper is a virus belonging to the genus *Morbillivirus* within the family *Paramyxoviridae*. The *canine distemper virus* (CDV) comprises a non-segmented, single-stranded, negative-sense RNA molecule (~15.7 kb). The genome encodes the envelope-associated protein M, two glycoproteins (hemagglutinin H and fusion protein F), two transcriptase-associated proteins (phosphoprotein P and large protein L), and the nucleocapsid protein N (van Regenmortel et al., 2000). The H protein is responsible for attachment to cell receptors in the first step of infection and promotes fusion activity of the F protein (Lamb and Parks, 2007). It has been reported that genetic variation in the H gene may favor the virus to avoid the immunological response generated by the "old strains" currently used in the vaccines (Bolt et al., 1997; Hirama et al., 2004; Iwatsuki et al., 1997, 2000). Analysis of the pronounced genetic diversity in the H gene permits the identification of different CDV geographic lineages. The basis for lineage identification is the level of amino acid diversity within and between different phylogenetic clusters: lineages have within-group amino acid diversity of less than 3.5% and between-group amino acid diversity greater than 4% (Bolt et al., 1997; Martella et al., 2006). There are currently eight lineages circulating throughout the world, denoted America 1, America 2, Asia 1, Asia 2, Europe 1, Europe 2 (Europe-wildlife), Europe 3 (Arctic like), and South Africa (An et al., 2008; Martella et al., 2006, 2007; Woma et al., 2010). In South America, Argentina is the only country that has reported the characterization of CDV within its borders. Genetic analysis based on partial amplification of the H gene suggested the existence of two circulating genotypes with different prevalences across the country (Calderon et al., 2007).

Here, we obtained the full-length H gene of two Argentine strains, each belonging to the CDV genotypes identified by Calderon et al. (2007), and five Uruguayan strains.

Total RNA was extracted from different types of samples collected from domestic dogs (Table 1). RNA from urine samples was extracted using the PureLink Viral RNA/DNA MiniKit (Invitrogen, Carlsbad, CA), while RNA from ocular discharge and clotted blood samples was extracted directly with TRIzol Reagent™ (Invitrogen).

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Table 1
The strains, country, accession number, sample origin, collection data (year), vaccination status, and clinical symptoms are listed. V, vaccinated; NV, not vaccinated; NS, nervous system; GIS, gastrointestinal system; RS, respiratory system; Conj, conjunctivitis.

Strain and country	Accession number	Sample origins	Year	Vaccination status	Clinical symptoms
Arg23 (Argentina)	FJ392652	Clotted blood	2003	V	NS and Conj
Arg24 (Argentina)	FJ392651	Clotted blood	2005	V	Conj
Uy102 (Uruguay)	JN215473	Urine	2007	V	GIS and RS
Uy109 (Uruguay)	JN215474	Ocular discharge	2008	NV	Unknown
Uy111 (Uruguay)	JN215475	Ocular discharge	2008	NV	GIS and RS
Uy128 (Uruguay)	JN215476	Urine	2009	NV	RS and Conj
Uy141 (Uruguay)	JN215477	Ocular discharge	2009	V	NS and RS

The complete H gene was amplified in two fragments of 1037 and 1227 bp using two pairs of primers (FE: 5′-CTTAGGGCTCA-GGTAGTCCAAC-3′; RI: 5′-GAGCGACAGGTATCACCTCTTC-3′; FI: 5′-GCTATCTCAGACGGAGTGTATGG-3′; RE: 5′-GTCGGTAAGGG ATTTCTCACCAC-3′). RT-PCR was carried out using the SuperScript One-Step RT-PCR kit (Invitrogen) according to the manufacturer's protocol followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 57 °C, and extension for 1 min at 72 °C. Amplicons were cloned into a pGEM®-T Easy Vector (Promega, Madison, WI) and sequenced bidirectionally with vector primers using an ABI prism 377-Perkin Elmer automated sequencer. The assembly of the full-length H gene was performed using DNAStar Lasergene software (DNAStar, Madison, WI). Nucleotide sequences were submitted to the GenBank database (Table 1).

For comparative analyses, a total of 47 CDV sequences from different geographic origins were used. These included the seven sequences characterized here and four Brazilian CDV strains available from the GenBank database. Nucleotide and deduced amino acid sequence alignments, p-distance calculation, and phylogenetic inferences were performed using MEGA 5.01 software (Tamura et al., 2007). The phylogenetic tree was constructed using the maximun likelihood approaches with the Tamura 3-parameter model (Tamura, 1992).

The H gene sequences obtained from Uruguayan and Argentine isolates presented an open reading frame of 1824 bp corresponding to a protein of 607 amino acids. Phylogenetic analysis showed that all South American strains except one formed a single clade with high bootstrap support (86%). This clade comprised all of the Uruguayan and Brazilian strains and one of the two analyzed strains from Argentina (Arg23) (Fig. 1). Strains included in this clade showed 2.2% amino acid divergence. This South American clade appeared related to the Europe 1 lineage with high bootstrap value (98%). These South American and European strains showed a level of divergence less than 2.7% between them and had a level of divergence above 4.3% in relation to the other CDV lineages. According to the criteria for defining a lineage, these South America strains should be considered as belonging to the Europe 1 lineage, which we suggest should be more correctly named the Europe 1/South America 1 lineage. The Europe 1/South America 1 lineage should now be considered one of the most widely distributed CDV lineages because it is present in Europe and in three South American countries. Previous studies of CDV strains from different regions have suggested that the genetic drift acting over the H gene drives geographic differentiation of the involved viruses (Martella et al., 2006). In this context, the genetic similarity between South American 1 and European CDVs may indicate a possible common origin of the viruses or a continuous genetic homogenization due to the commercial exchange between these regions.

One single South American strain from Argentina (Arg24) appeared separated in the phylogenetic tree, it can be considered representative of a new lineage (South American 2) because it differed from other lineages in more than 4.0% of its amino acids. Interestingly, Calderon et al. (2007) reported that the genotype, including the Arg24 strain, is the prevalent genotype in Argentina (23 of 24 isolates). Accordingly, the South America 2 lineage may be distributed widely in Argentina but not in Uruguay and Brazil. Although supported by a lower bootstrap value (61%), the South America 2 lineage appeared related to the wildlife CDV derived from the Europe 2 (Europe-wildlife) lineage (Fig. 1). This relationship suggests that the South America 2 lineage may also be a lineage circulating in wild populations in South American countries. This idea is supported by the recent genetic characterization of a partial H gene sequence from wild carnivore CDVs in Argentina that showed a close relationship with the Arg24 strain belonging to the South America 2 lineage (Ferreyra et al., 2009). Moreover, the South America 2 lineage has a wildlife-specific residue in the H protein that is involved in determining host-cell tropism *in vitro* (von Messling et al., 2005). Most CDV strains from dogs have 530E or 530G, whereas the majority of CDV strains from non-dog hosts have R, D, or N residues (McCarthy et al., 2007). Wild-type strains described by Ferreyra et al. (2009) and strains from the South America 2 and Europe 2 lineages share 530D, whereas strains belonging to the Europe 1/South America 1 lineage have 530G. This evidence suggests that the South America 2 lineage may have been transferred from wild carnivores to domestic dogs. Alternatively, the South America 2 lineage may have been transferred to wild carnivores by dogs, and the differences observed may be the consequence of the adaptation to a new host (Ferreyra et al., 2009). Although this lineage was detected only in Argentina, we cannot rule out the possibility that it is not present in wildlife carnivores of other South American countries. This must be confirmed by extending genetic studies to more samples and additional carnivore hosts.

Phylogenetic analysis also revealed that all South American strains are clearly separated from the vaccine strains (Fig. 1). Amino acid sequence comparison showed high variability between field and vaccine strains (from 7.8 to 8.9% amino acid sequence). These vaccine viruses were isolated in the 1950s, and they have been successfully used for CDV control (Demeter et al., 2010). However, in the last two decades, there have been several reports of a resurgence of CDV in vaccinated dog populations (Blixenkrone-Møller et al., 1993; Calderon et al., 2007; Decaro et al., 2004; Gemma et al., 1996). These outbreaks may result from insufficient attenuation of the vaccine virus or by the emergence of new field strains with the ability to evade the immune response generated by current vaccines (Keawcharoen et al., 2005; Pardo et al., 2005). Our data show that vaccinated dogs were affected by field strains of CDV, disputing the hypothesis of a vaccine viral reversion. It remains unclear if the effectiveness of the currently employed vaccines may be partially compromised by the extent of observed genetic/antigenic variation.

In summary, our data indicate the presence of two co-circulating lineages with different prevalence in South America: Europe 1/South America 1 and the new South America 2 lineage. The level of genetic variation observed between these lineages and vaccine strains should be considered a possible factor leading to the resurgence of distemper cases in vaccinated dog populations.

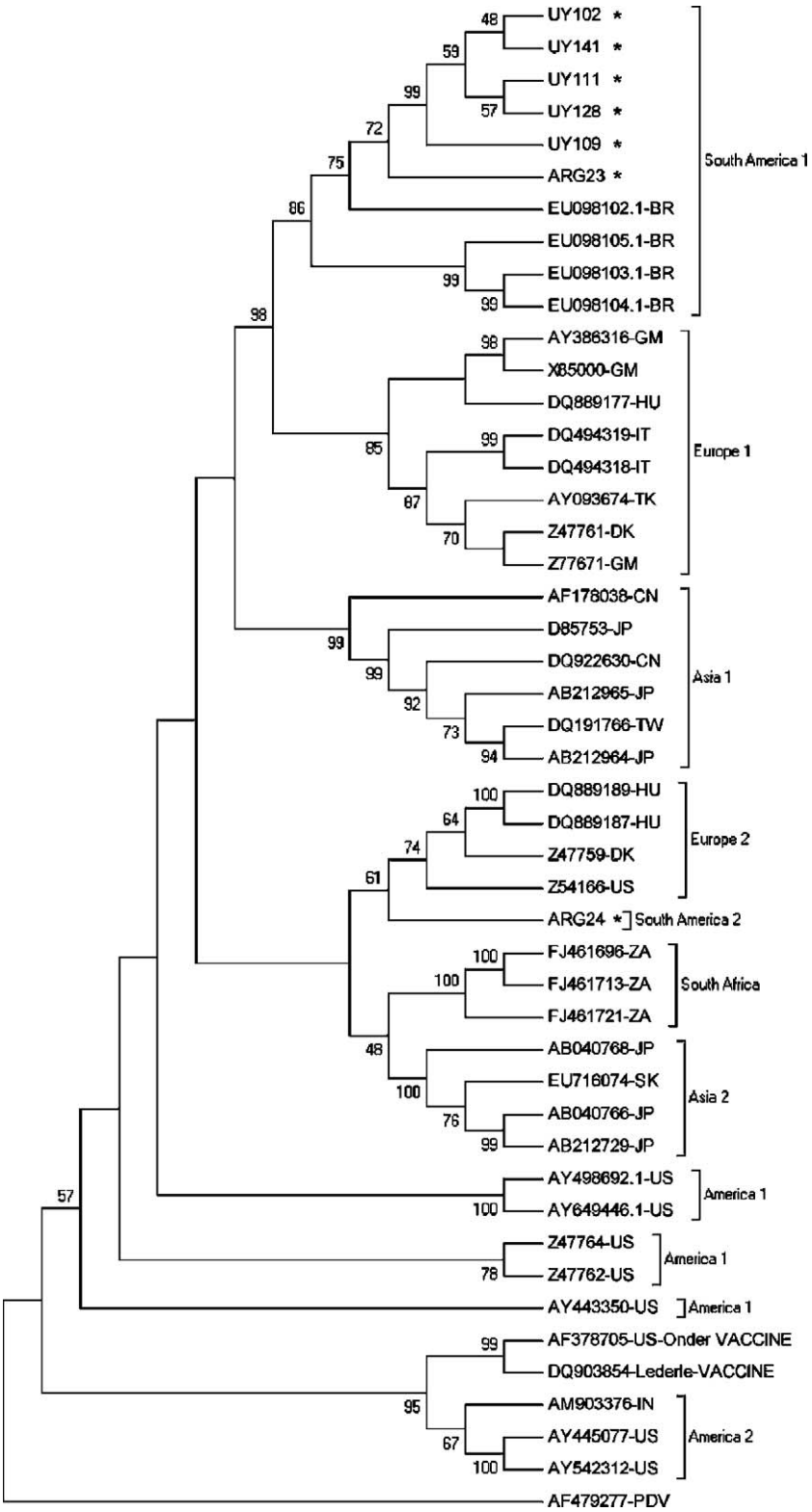

Fig. 1. Phylogenetic relationships among the 47 CDV strains belonging to the different lineages based on the alignment of the nucleotide sequence of the H gene. The GenBank accession numbers, countries, and lineages are indicated. The South American strains characterized here are listed with their names and marked with asterisks. ARG, Argentina; BR, Brazil; DK, Denmark; CN, China; GM, Germany; HU, Hungary; IT, Italy; JP, Japan; TW, Taiwan, TK Turkey, SK, South Korea, US, United States of America; UY, Uruguay; and ZA, South Africa.

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