PHYLOGENETIC ANALYSIS OF WILD-TYPE CANINE DISTEMPER VIRUSES CIRCULATING AMONG DOGS FROM THE ABURRÁ VALLEY, COLOMBIA

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DEDICATORIA

Les dedico estas páginas como un símbolo del gran esfuerzo que nos unió como familia en estos últimos tres años, llenos de desafíos de vida y no solo de ciencia. Vencimos.

A mi Madre

A mi Padre

A mi Hermana

A Luna

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"Este trabajo está especialmente dedicado a los pacientes y sus familias"

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INDEX OF ABBREVIATIONS

Asp: Asparagine

BLAST: Basic Local Alignment Search Tool

CAV-2: Canine adenovirus- 2

CDV: Canine distemper virus

CNS: Central Nervous System

DL: Demyelinating leukoencephalomyelitis

ELISA: Enzyme-linked immunosorbent assay

F: Fusion protein

Gly: Glycine

GTR + G: General time-reversible model plus gamma distribution rate heterogeneity

H: Hemagglutinin

EpR: Epithelial putative receptor

L: Polymerase

M: MAtrix

ML: Modified live

MV: Measles Virus

mRNA: Messenger ribonucleic acid

N: Nucleocapsid

P: Phosphoprotein

PCR: Polymerase chain reaction

pi: Post-infection

rCDV: Recombinant CDV

RNA: Ribonucleic acid

RT-PCR: Reverse transcriptase polymerase chain reaction

SLAM: Signaling Lymphocyte-Activation Molecule

TVM + G: Transversional model plus gamma distribution rate heterogeneity

Abstract

Canine Distemper Virus (CDV) is a highly contagious viral disease of carnivores, affecting both wild and domestic populations. The hemagglutinin (H) gene, encoding for the attachment protein that determines viral tropism, shows high heterogeneity among strains, allowing for the distinction of ten different lineages distributed worldwide according to a geographic pattern. We obtained the sequences of the full-length H gene of 15 wild-type CDV strains circulating in domestic dog populations from the Aburrá Valley, Colombia. A phylogenetic analysis of H nucleotide sequences from Colombian CDV viruses along with field isolates from different geographic regions and vaccine strains was performed. Colombian wild-type viruses formed a distinct monophyletic cluster clearly separated from the previously identified wild-type and vaccine lineages, suggesting that a novel genetic variant, quite different from vaccines and other lineages, is circulating among dog populations in the Aburrá Valley (Colombia). We propose naming this new lineage as "South America 3". This information indicates that there are at least three different CDV lineages circulating in domestic and wild carnivore populations in South America. The first one, renamed Europe/ South America 1, circulates in Brazil and Uruguay; the second, South America 2, appears to be restricted to Argentina; and the third, South America 3, which comprises all the CDV strains characterized in this study.

Resumen

El virus del Distemper Canino (CDV) es el agente causal de una enfermedad altamente contagiosa que afecta a poblaciones caninas domésticas y silvestres. El gen de la hemaglutinina (H), la proteína de membrana que determina el tropismo viral, presenta gran variabilidad genética entre las cepas existentes, identificándose hasta la fecha diez linajes distintos distribuidos alrededor del mundo. Fue posible obtener la secuencia completa del gen H de 15 cepas circulantes en las poblaciones caninas domésticas del Valle de Aburrá (Departamento de Antioquia, Colombia). Se realizó un análisis filogenético de las secuencias nucleotídicas de las cepas de CDV colombianas con otros virus de CDV aislados en diferentes partes del mundo, incluyendo las cepas vacunales. Las cepas colombianas se agruparon monofiléticamente y por fuera de los linajes previamente descritos. Los anteriores resultados sugieren que en las poblaciones caninas domésticas del valle de Aburrá circula una variante genética del virus del distemper canino, no reportada anteriormente en ningún país y altamente divergente de las cepas vacunales y otros linajes reportados hasta el momento. Proponemos que este nuevo linaje sea llamado "Sur América 3". Esta información sugiere que en la actualidad circulan al menos tres linajes distintos en las poblaciones Suramericanas de carnívoros domésticos y silvestres. El primero de ellos, re-nombrado como Europa/Suramérica 1, circula en Brasil y Uruguay; el segundo, conocido como Suramérica 2, parece estar restringido a la Argentina; y un tercero, Suramérica 3, que alberga todas las cepas de CDV caracterizadas en este estudio.

Palabras clave: *cepas silvestres, filogenia, gen de la hemaglutinina (H), genotipo, RT-PCR.*

 INTRODUCTION

Canine distemper virus (CDV) is the etiological agent of a highly prevalent viral infectious disease of carnivores, posing a conservation threat to endangered species around the world (Appel, 1987; McCarthy et al., 2007). CDV belongs to *Paramyxoviridae* family, genus *morbillivirus*, which includes viruses with epidemiologic relevance in human and animal populations such as *Measles virus*, *Phocine distemper virus*, *peste-des-petits-ruminants virus*, and *Rinderpest virus*, the latter already eradicated (Mariner et al., 2012). CDV is an enveloped, negative-sense, single-stranded RNA virus with a diameter between 150 and 300 nm and a genome length of 15,690 nucleotides, organized into six contiguous, non-overlapping, transcription units encoding for six structural proteins, known as nucleocapsid (N), phosphoprotein (P), matrix (M), fusion protein (F), hemagglutinin (H), and polymerase (L), protein (Barret, 2010; Lamb and Parks, 2007; Sidhu et al., 1993).

The host range of CDV comprises all families within the order Carnivora*,* and it has recently expanded to non-human primates (Deem et al., 2000; Qiu et al., 2011; Sakai et al., 2013; Sun et al., 2010; Yoshikawa et al., 1989). Clinical symptoms in all affected species are influenced by strain virulence, environmental conditions, host age and host immune status. The gastrointestinal tract, respiratory and nervous systems are most affected in all species (Deem et al., 2000). CDV is consider a immunopreventable disease since the release of the first vaccines in the 1950´s. These vaccine strains are still in use in immunoprophilactic programs worldwide; however, since the 1990´s there have been reports of disease in vaccinated dogs, raising concerns about the efficacy of the immune response elicited by vaccine strains against field strains.

The H protein is of paramount importance because it determines viral tropism *in vivo* and *in vitro* and an effective immune response against it protects the host from infection (Martella et al., 2008; Woma et al., 2010). Several comparative studies have revealed that the H gene is subjected to a higher genetic variability

(approximately 10%) when compared to the other CDV genes, (Barrett, 1987; Haas et al., 1999) which makes it suitable for lineage identification and genetic analysis. Phylogenetic studies based on the complete sequence of the H gene from several CDV strains isolated in distinct geographic locations around the world have revealed a genetic/antigenic drift explained by a geographic pattern. According to this pattern, there are ten distinct lineages known as America 1 (that includes almost all of the commercially available vaccines), America 2, Artic-like, Rock born Like, Asia 1, Asia 2, Africa 1, European Wildlife, Europe/South America 1 and South America 2 (Blixenkrone-Møller et al., 1992; Calderon et al., 2007; Haas et al., 1997; Harder et al., 1996; Harder et al., 1993; Iwatsuki et al., 2000; Orvell et al., 1990; Panzera et al., 2012; Woma et al., 2010).

Recent studies based on the nucleotide sequence of the hemagglutinin gene of CDVs from Uruguay, Argentina and Brazil have revealed two co-circulating lineages of CDV with different prevalences in South America. The most prevalent lineage belongs to the Europe lineage, renamed by the authors as Europe/South America 1 (Panzera et al., 2011). The other lineage, known as South America 2, is restricted to Argentinian canine populations; it shows a clear amino acid divergence with the previously identified lineages and appears to be related to strains isolated form wild carnivore species in Europe (European Wildlife lineage) (Calderon et al., 2007; Panzera et al., 2011). So far, there is no available information regarding lineage circulation dynamics of CDV in countries located in the northern part of South America (ie. Colombia, Venezuela, Ecuador, and the Guiana's) and whether or not the known South American lineages are also prevalent in this part of the continent. In this study we provide the first genetic evidence of CDV in Colombia. Additionally, we phylogenetically characterized 15 wild-type CDV strains circulating in domestic dog populations from Colombia and described the main clinical features in the animals affected by the disease, concluding that these strains belong to a distinct new lineage, different from those reported previously. This information suggests that there are at least 3 different CDV lineages circulating in domestic and wild carnivore populations in South America.

OBJECTIVES

General objectives:

To confirm the circulation and transmission of CDV among dogs in the Aburrá Valley, department of Antioquia, Colombia.

To characterize phylogenetically CDVs isolated in the study area in order to establish genetic lineages

Specific objectives:

- To confirm CDV infection by RT-PCR in dogs with a presumptive CDV diagnosis.

- To perform a phylogenetic analysis of the full-length H gene in samples from dogs confirmed as CDV positive by RT-PCR.

- To describe the main clinical features in those animals where CDV infection was confirmed by RT-PCR.

1. LITERATURE REVIEW

1.1 Viral characteristics and taxonomy

Canine distemper virus (CDV) belongs to *Morbillivirus* genus within the *Paramixoviridae* family which includes *Phocine distemper virus*, *Measles virus* (MV), *peste-des-petits-ruminants virus*, and *Rinderpest virus* (King AMQ et al., 2012).

CDV is an enveloped, negative-sense, single-stranded RNA virus with a diameter ranging between 150 and 300 nm and a genome length of 15,690-bp, organized into six contiguous, non-overlapping, transcription units encoding for six structural proteins, known as nucleocapsid (N) which is an RNA-binding protein, a phosphorotein (P), a matrix protein (M), a fusion protein (F), the hemagglutinin protein (H), and a polymerase protein (L) (Figure 1) (Barret, 2010; Lamb and Parks, 2007; Sidhu et al., 1993). CDV transcriptional units are separated by untranslated regions (UTRs), which are relatively uniform in length, with the exception of the UTR between the matrix (M) and fusion gene (F) (Anderson et al., 2012).

The N, P and L proteins along with the genome constitute the transcription/replication unit of the virus, the ribonucleoprotein core. The surface glycoproteins H and F are embedded in the host-derived lipid envelope; the first one is an adhesion glycoprotein that exhibits hemagglutinin activity once it binds the Signaling Lymphocyte-Activation Molecule (SLAM) in the host cell, and the second one mediates viral penetration through membrane fusion of viral envelope and host cell plasma membrane (Barret, 2010; Samal, 2010). The H protein is essential for viral tropism either *in vivo* or *in vitro* and an effective immune response against it protects the host from infection (Martella et al., 2008; Womma et al., 2010).

The M protein, which constitutes the internal layer of the viral envelope, interacts with the cytoplasmic domains of the membrane associated proteins H and F and also with ribonucleoproteins formed in the cytoplasm during the virus

replication cycle, hence putting together the two essential components of the budding virion, and playing and essential role in virus budding. Recently, it has been described that the viral genome also encodes for two non-structural proteins known as C and V, which are encoded in the P gene transcription unit and have functions related to transcription control and replication and also play an important role in virus evasion of the host's innate immune responses (Samal, 2010; von Messling et al., 2006).
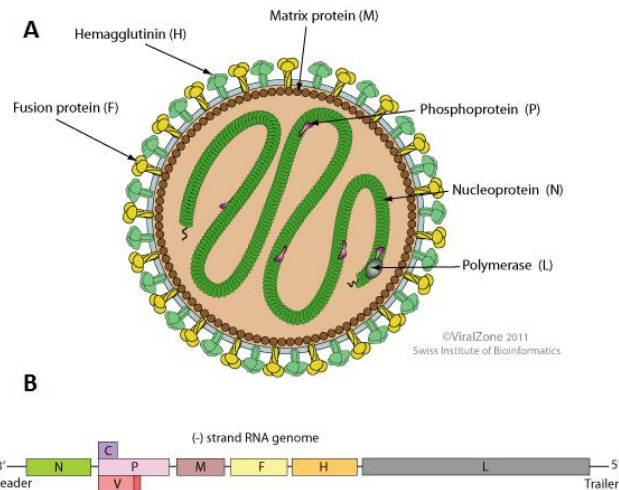
Figure 1. CDV infectious virion and genome organization. A) Schematic diagram of a CDV particle in cross section: N, nucleocapsid; P, phosphoprotein; L, polymerase protein; M, matrix or membrane protein; F, fusion protein; N, hemagglutinin. In green the viral RNA. B). Map of genomic RNA (3' to 5') of CDV. Each box represents a separately encoded mRNA; multiple distinct ORFs within a single mRNA are indicated in overlapping boxes on P. (Reprinted from: http://viralzone.expasy.org)

1.2 Replication Cycle

Morbillivirus gene expression and RNA replication occur in the cytoplasm of infected cells, and progeny virions bud from the plasma membrane (Figure 2). Field strains of *Measles virus* use SLAM and an epithelial putative receptor known as KeR to bind lymphocytes and epithelial cells, respectively (Erlenhoefer et al., 2001; Leonard et al., 2008; Tatsuo et al., 2001; Tatsuo and Yanagi, 2002). Site directed mutational analysis in CDV mapped several residues in the H protein that are necessary for efficient SLAM and KeR-dependent membrane fusion (Langedijk et al., 2011; von Messling et al., 2005).

After receptor binding, the F protein mediates fusion of the viral envelope and the plasma membrane of the host cell. Subsequently, viral nucleocapsid is released into the cytoplasm and initiates transcription. The viral polymerase enters at the promoter located at the 3´-end of the genome which serves the dual function of mRNA and antigenome synthesis. Transcription is linear, sequential, and involves a stop-start mechanism guided by the gene-start and gene-end signals. Viral mRNAs are 5´-capped by the viral polymerase and contain a 3´ poly(A). Intracellular accumulation of viral nucleocapsid-associated proteins stimulates the initiation of genome replication which is tightly linked to encapsidation of the progeny virus. Afterwards, the M protein assembles the virion by forming a bridge between the cytoplasmic tails of envelope proteins and the nucleocapsids. Both, the final assembly and budding of the virus occur at the plasma membrane of infected cells (Figure 2) (Samal, 2010).
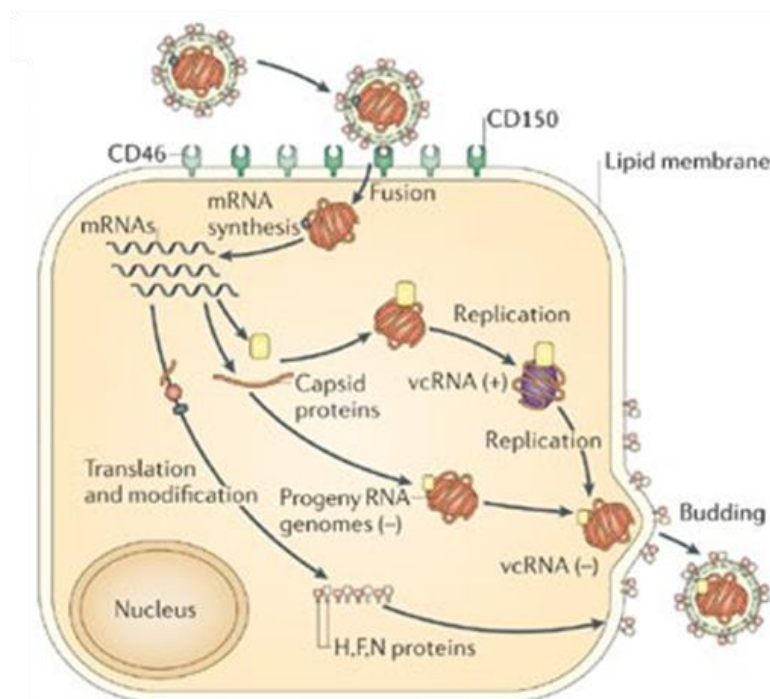
Figure 2. Replication cycle of *Measles virus*. Reprinted from Moss and Griffin, 2006.

1.3 Host range: from dogs to wild life

CDV is the most important infectious disease of domestic dogs (*Canis familiaris*) worldwide, and its fatality rate is second only to that of rabies (Swango, 1995). Evidence of CDV infection has been reported in all families of terrestrial carnivores, becoming a conservation threat to endangered species around the world (Appel, 1987; Deem et al., 2000; McCarthy et al., 2007).

Morbidity and mortality vary greatly in carnivores and clinical signs are influenced by strain virulence, environmental conditions, host´s age and immune status. The gastrointestinal tract, respiratory and nervous systems are most affected in all species (Deem et al., 2000).

Within *Canidae* family, the domestic dog (*Canis familiaris)* is the species with more reports of occurrence of CDV and it is estimated that 70% of all infections are subclinical; however the acute generalized form produces high mortality rates (Blixenkrone-Moller et al., 1993; Decaro et al., 2004; Green and Appel, 2006).

A serologic investigation of CDV in wolves (*Canis vulpes*) of the United States and Canada found a 29% seroprevalence of CDV antibodies, and there appeared to be a direct relationship between pup mortality and CDV antibody titers in adult pack members. (Johnson et al., 1994). The Australian dingo (Armstrong and Anthony, 1942), red foxes (Little et al., 1998) and coyotes (Cypher et al., 1998) are also among the susceptible species to CDV.

A phylogenetic analysis of fragments of the P and F genes of a CDV strain that caused a fatal infection in a pack of African wild dogs in the Serengeti National Park in 2007 (Goller et al., 2010) could establish that the pack was infected with a variant that showed high homology with two variants from a fatal CDV infection of captive African wild dogs in the Nkomazi Game Reserve in 2000 (van de Bildt et al., 2002) and a CDV epidemic in lions, spotted hyenas and bat-eared foxes in the Serengeti National Park in 1993 and 1994 (Carpenter et al., 1998; Haas et al., 1996; Roelke-Parker et al., 1996).

The host rage of CDV comprises other families such as Viverridae (Chandra et al., 2000; Machida et al., 1992) and Mustelidae; the latter includes several reports of infection in ferrets, skunks, weasels and minks (Goodrich et al., 1994; Williams et al., 1988). CDV is endemic in the United States raccoon population (family Procyonidae) that currently constitutes an important reservoir of the virus for domestic dogs and zoo animals (Deem et al., 2000; Roscoe, 1993). The susceptibility of the Ursidae family to CDV has been established through serological surveys (Chomel et al., 1998; Follmann et al., 1996; Mainka et al., 1994), and there is also a report of CDV infection in a giant panda from the Republic of China (Guo et al., 2013; Qiu and Mainka, 1993).

To date there is no scientific evidence of CDV infection in domestic cats and the experimental inoculation of this species with the highly virulent strain Snyder Hill only yielded an asymptomatic infection without evidence of viral shedding (Gaskin, 1974). On the other hand, there have been sporadic reports of CDV infection in large felids such as lions, panthers, leopards and tigers (Appel et al., 1994; Harder et al., 1996; Roelke-Parker et al., 1996). A serosurvey of CDV in jaguars, pumas and ocelots in the Brazilian Atlantic jungle reported for the first time the exposure of wild felids to CDV in this country (Nava et al., 2008). In 1996 a CDV strain closely related to the one that caused high mortality in sympatric lions caused an outbreak of CDV in a group of spotted hyenas (*Crocuta crocuta, family* Hyaenidae) in the Serengeti (Haas et al., 1996).

During spring of 2000, a mass mortality of Caspian seals (*Phoca caspica*), recognized as a vulnerable species by the International Union for the Conservation of Nature (IUCN, 1996), was attributed to CDV infection, possibly transmitted by direct contact with other carnivore species of the same region (Kennedy et al., 2000). Recently, Clancy et al. (2013) reported the prevalence of CDV antibodies in different captive U.S. phocids in the United states, finding seroprevalences of 25.5% and 25.0% for grey seals *(Halichoerus grypus)* and harbor seals *(Phoca vitulina),* respectively.

In 1989, the first case of natural infection of CDV in non-human primates, specifically Japanese monkeys (*Macaca fuscata*), was documented (Yoshikawa

et al., 1989). In 2006, a serious outbreak of CDV affected 10,000 animals in a primate breeding farm in the Guangxi Zhuang Autonomous Region in southern China. The morbidity rate in young monkeys was 60%, with approximately 30% death rate; on the other hand, morbidity and death rates for adults were 25% and 5%, respectively (Qiu et al., 2011). The disease spread throughout China, particularly to experimental animal facilities in Wuhan, Kunming, and Beijing. In the Laboratory Animal Center of the Academy of Military Medical Science in Beijing, the animals affected (*Macaca mulatta*) exhibited respiratory signs, anorexia, fever, thicken of footpads and facial rash. The high mortality rate (60%) suggested an increase of CDV virulence for this species (Sun et al., 2010). The disease was also introduced into a few wildlife parks in China, but further spread has not been reported, perhaps because of the low population density of susceptible animals in these locations (Qiu et al., 2011). In 2008, a CDV outbreak in a Japanese experimental animal facility involved several Cynomolgus monkeys (*Macaca fascicularis*) imported from China. A phylogenetic analysis of the whole genome sequence from one of the affected animals showed that this particular strain was closely related to the CDV strains associated with the recent outbreaks in rhesus monkeys in China, suggesting continuing chains of CDV infection in monkeys (Sakai et al., 2013). This particular expansion in CDV´s host range is quite concerning, especially considering new experimental studies that demonstrate that only one amino acid change in the H-protein at position D540G is required for functional adaptation to the human SLAM receptor (Bieringer et al., 2013). In this context, Bieringer et al. (2013) hypothesize that CDV might eventually cross the species barrier to humans and emerge as a new human pathogen when measles eradication goal is already achieved and vaccination campaigns are stopped.

1.4 Genetic diversity and epidemiology of CDV

CDV has been described as a monotypic virus by monoclonal antibodies and a single exposure confers long term immunity; however, there are different strains that differ in virulence and tropism, situation evidenced by their differential pathogenic patterns (Blixenkrone-Moller et al., 1993; Summers et al., 1984). Epidemiological studies based on molecular techniques constitute an essential

tool to study circulation dynamics of different strains in susceptible hosts. Several comparative studies have revealed that the H gene is subjected to a higher genetic variability (approximately 10%) compared to the other genes; for example, the P gene varies approximately 4%, which is within the variability range of the other CDV genes (Barrett et al., 1987; Haas et al., 1999). This variability may affect some specific sites on the H protein that are related with immune neutralization, therefore generating an important epitope disruption that could have consequences on the infection status of the host and the clinical outcome. In recent years a pronounced genetic diversity has been reported in CDV isolations around the world and several episodes of CDV were confirmed in vaccinated animals. This evident variability could alter the antigenic profile of these new genetic variants when compared to vaccine strains (Blixenkrone-Møller et al., 1993; Ek-Kommonen et al., 1997; Iwatsuki et al., 2000; Martella et al., 2006; Martella et al., 2008; Uema et al., 2005).

A lineage is characterized at the level of amino acid divergence within and between different phylogenetic clusters. Within group amino acid divergence is known to be less than 3.5%, whereas amino acid divergence between groups is greater than 4% (Bolt et al., 1997; Martella et al., 2006). Phylogenetic studies based in the complete sequence of the H gene of several CDV strains isolated in distinct geographic sites around the world have revealed a genetic/antigenic drift explained by a geographical pattern. According to this, seven distinct lineages (clusters or genotypes) were reported until 2007. These are known as America 1 (includes almost all the commercially available vaccines), America 2, Artic-like, Asia 1, Asia 2, Europe, and European Wildlife (Blixenkrone-Møller et al., 1992; Haas et al., 1997; Harder et al., 1996; Harder et al., 1993; Iwatsuki et al., 2000; Orvell et al., 1990).

In 2007, a phylogenetic study of partial nucleotide sequences of the H gene of vaccinated and non-vaccinated dogs from Argentina found a new lineage denominated "Argentina" (Calderon et al., 2007) which forms a completely separate clade. In 2012, a new phylogenetic study with full-length H gene sequences from South American dogs found that all Uruguayan, Brazilian and one Argentinean CDV strain belonged to the already known Europe lineage

(Figure 3), suggesting a possible common origin of the viruses explained by a continuous genetic homogenization due to the commercial exchange of dog breeds between these regions (Panzera et al., 2012).

Surprisingly, one Argentinean strain which represents the prevalent genotype in this country (Calderon et al., 2007), appeared separated in the phylogenetic tree, showing an amino acid divergence above 4% when compared with other H sequences reported around the world; however, it appeared to be related to sequences belonging to the European wildlife lineage, although with low bootstrap support (61%); therefore, the authors proposed to call this new genotype as South America 2 (Panzera et al., 2012). Recently, a study describing the phylogenetic characteristics of wild-type strains of CDV of dogs from northern Paraná, confirmed that Brazilian strains have a European origin (Negrao et al., 2013).

In 2010, Woma et al. isolated and characterized, for the first time in the African continent, several wild-type CDV strains from domestic dogs. Phylogenetic analysis of the full-length H gene showed that all South African viruses formed a distinct cluster with high posterior probability. On the other hand, Gamiz et al., 2011 performed an analysis of partial-length H gene sequences (509 bp) of dogs from Jalisco and Mexico states, concluding that there might be a new American CDV genotype circulating among dog populations in Mexico. Considering that this analysis is based on partial sequences, the question remains about whether or not these findings are comparable with the recent phylogenetic studies that include full-length H sequences.
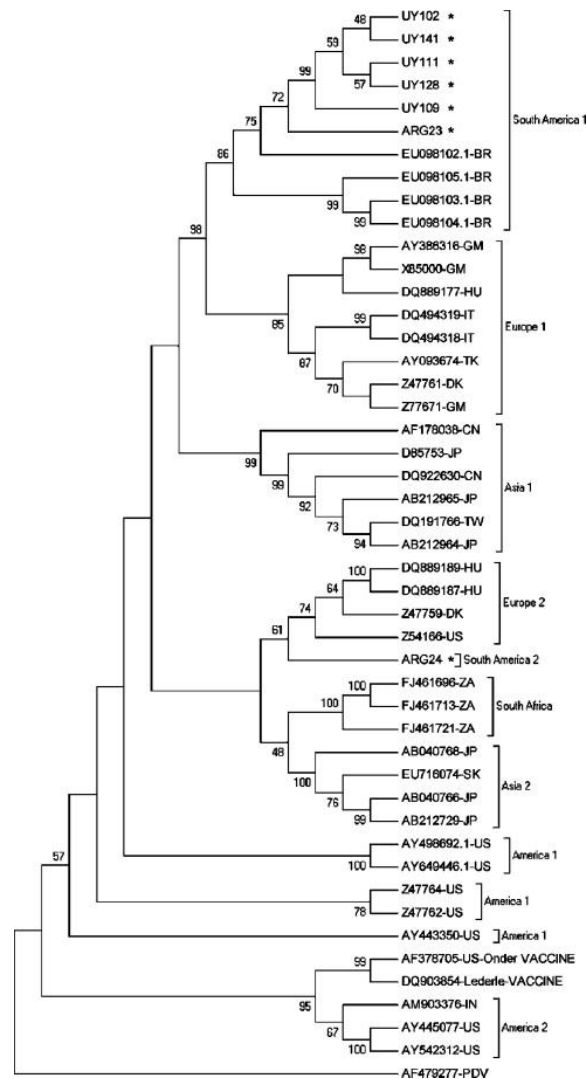
Figure 3. Lineages of Canine distemper virus. Phylogenetic relationships among the 47 CDV strains belonging to the different lineages based on the alignment of the nucleotide sequence of the H gene. The GenBank accession numbers, countries, and lineages are indicated. ARG, Argentina; BR, Brazil; DK, 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.

Reprinted from: Panzera Y, Calderón MG, Sarute N, Guasco S, Cardeillac A, Bonilla B, Hernández M, Francia L, Bedó G, La Torre J, Pérez R. Evidence of two co-circulating genetic lineages of canine distemper virus in South America. Virus Res 2012; 163:401-404.

1.4.1 CDV transmission

Virus shedding starts at 7 days post infection (pi) and can last up to 60-90 days pi. Although CDV is most abundant in respiratory exudates and spreads by aerosol or droplet exposure, it can also be isolated from other body tissues and secretions, including urine. Contact among susceptible populations (ie, puppies) and recently infected dogs with clear signs of disease or subclinically infected, maintains the virus in a population (Green and Appel, 2006).

CDV is a monotypic virus, as defined by monoclonal antisera, and exposure to virulent CDV confers long term immunity (Green and Appel, 2006; Martella et al., 2008; Martella et al., 2007); however, vaccine related immunity is not absolute and dogs that do not receive periodic immunizations may lose their protection and become infected after periods of stress, immunosuppression or contact with diseased animals (Green and Appel, 2006).

Infection rate is higher than the disease rate, reflecting a certain degree of natural and vaccine-induced immunity in domestic dog populations. It is estimated that 25-75% of susceptible individuals become infected but are capable of clearing the virus from their bodies without any signs of disease. Although most dogs that recover clear the virus, some of them may harbor CDV in their Central Nervous System (CNS) (Green and Appel, 2006).

Age related susceptibility to CDV (puppies between 3 and 6 months of age) correlates with the loss of maternal antibodies after weaning. In contrast, in isolated and susceptible populations, the disease is severe and widespread, affecting dogs of all ages. This could be explained by the lack of pre-existing immunity against the disease (Green and Appel, 2006).

1.5 CDV Pathogenesis

There is a great variation regarding disease duration, severity and clinical signs, both in dogs experimentally and naturally infected by CDV. The incubation period may vary from 1 to 4 weeks and it depends on viral strain, age of the host at the time of infection and immune status of the animal. Clinical manifestations may range between virtually no sings to extreme severity of the disease, including respiratory, digestive and CNS involvement, reaching mortality rates of approximately 50% (Beineke et al., 2009).

1.5.1 Route of infection and virus spread

During natural exposure, dogs become infected by inhalation of airborne viruses or via infective aerosol droplets (Krakowka et al.,1980). CDV reaches the upper respiratory tract and replicates in tissue macrophages within 24 hours, and spreads in these cells by the lymphatic system to tonsils and bronquial lymph nodes where the virus load increases from days 2 to 6 pi, due to active replication (Appel, 1969). After this compartmental replication, CDV is disseminated via lymphatic vessels and blood to distant lymphoid organs during the first viremic phase (Beineke et al., 2009). This widespread virus proliferation in lymphoid organs corresponds to an initial rise in body temperature accompanied by leucopenia, specifically lymphopenia due to direct damage to lymphoid cells (T and B cells) by days 3 to 6 pi (Green and Appel, 2006) (Figure 4).

Extensive T and B cell damage can be explained by the ability of the H protein to bind the CD150 receptor (SLAM) on the surface of lymphocytes, situation that may account for CDV´s specific lymphotropism. *In vitro* studies have shown that T and B cell lines actively expressing SLAM molecules, experience apoptosis after being infected by the virus (Beineke et al., 2009; Green and Appel, 2006).

Further spread of CDV to epithelial and CNS tissues occurs between days 8 to 9 pi, and it probably occurs hematogenously as cell-associated or plasma

phase viremia but depends on the humoral and cell-mediated immune status of the host. At the time of epithelial colonization virus shedding starts and can be detected in all body excretions and secretions, even in dogs with subclinical disease. By day 14 pi, dogs with an adequate humoral and cell-mediated immune response clear the virus from most tissues and show no clinical signs of illness (Figure 4). Dogs with intermediate levels of cell-mediated immunoresponsiveness and delayed antibody titers by days 9 to 14 pi experience virus spread to epithelial tissues. As antibody titers increases, clinical signs may resolve and the virus can be cleared of most body tissues, but it may persist for long periods in uveal tissues, neurons and integument (Appel et al.,1982; Winters et al., 1983).

In dogs with poor immune status, CDV spreads between days 9 to 14 pi to exocrine and endocrine glands, skin, and epithelium of the gastrointestinal, respiratory and urinary tract, giving rise to severe clinical signs usually exacerbated by secondary bacterial infections. In these animals virus persist in their tissues until death (Appel et al., 1984; Green and Appel, 2006; Winters et al., 1983) (Figure 4).
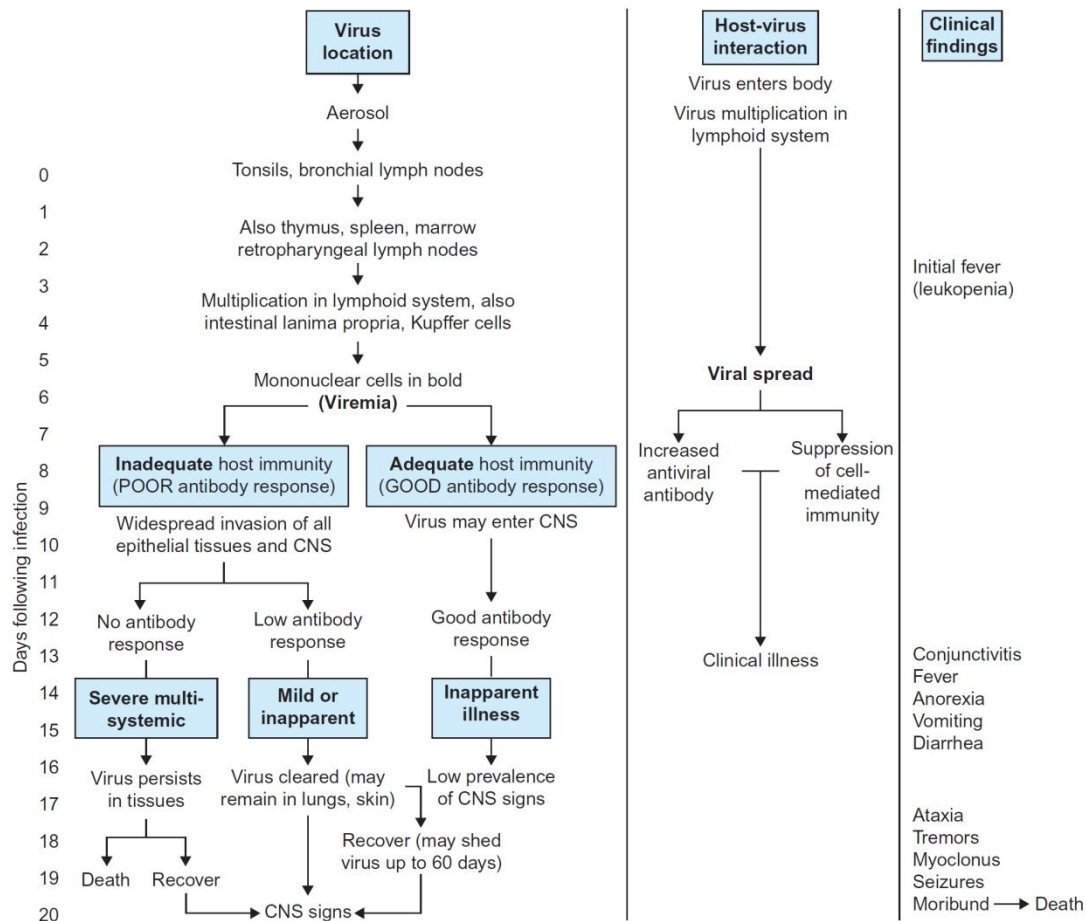
Figure 4. Sequential pathogenesis of canine distemper.

Adapted from: Green EC, Appel MJ. Canine distemper virus. In: Green, editor. Infectious diseases of the dog and cat. St Louis: Saunders; 2006.

1.5.2 Route of entry and viral spread in CNS

Neurologic invasion occurs predominantly via the hematogenous route (Krakowka, 1989; Krakowka et al., 1987). Some studies indicate that CDV may spread along the cerebrospinal fluid (CSF) pathway thus infecting ependymal and subependymal white matter (Vandevelde et al., 1985). The leukocyte-associated viremia represents the main source of hematogenous infectivity (Rockborn, 1958; Summers et al.,1979). Viral antigen is first detected within CNS capillaries and venular endothelia at 5 and 6 days pi and/or in perivascular lymphocytes, astrocytic foot processes and pericytes at 8 days pi (Beineke et

al., 2009; Krakowka et al., 1987; Summers et al., 1987). Moreover, a productive CDV infection of the choroid plexus epithelium with release of progeny virus into the cerebrospinal fluid followed by ependymal infection and spread of the virus to the subependymal white matter can be observed at 10 days pi (Appel and Gillespie, 1972; Higgins et al., 1982; Vandevelde et al., 1985).

Canine distemper encephalitis can be classified in different subtypes according to morphological changes and brain areas affected. Generally, a polio- and a leukoencephalitis, can be distinguished (Beineke et al., 2009). There are numerous factors that determine the type of lesion produced by the virus in the CNS and the course of infection, such as age and immunocompetence of the host at the time of infection, neurotropic and immunosuppressive properties of the viral strain and/or time at which lesions are examined (Bernard et al., 1993; Krakowka and Koestner, 1976; Pearce-Kelling et al., 1990; Raw et al., 1992; Summerset al., 1984).

1.5.3 Clinical manifestations

At day 10 pi dogs may develop respiratory, gastrointestinal and dermatologic signs that reflect the epithelial localization of the virus at this stage of the disease. These signs are commonly exacerbated by secondary bacterial infections and include purulent nasal discharge, coughing, dyspnea, pneumonia, diarrhea, vomiting, and dermal pustules (Figure 4). Patients that survive subacute and subclinical infections develop enamel hypoplasia and hyperkeratosis of the foot pads and nose (Figure 5) (Green and Appel, 2006).

From day 20 pi, it is possible to observe neurologic signs that vary according to the area of the CNS affected by the virus (Figure 4). These neurologic complications are often progressive, and affect prognosis and recovery from infection. Although signs related with meningeal inflammation such as hyperestesia and cervical or paraspinal rigidity can occur, parenchymal signs predominate in CDV infected animals. Seizures, cerebelar and vestibular signs, paraparesis or tetraparesis with sensory ataxia and myoclonus are common. The types of seizures exhibited by the patient are related with the region of the

forebrain affected by the virus. The "chewing gum" type of seizures, classically associated with CDV infection, is a clinical manifestation of polioencephalomalacia of the temporal lobes. It is possible to observe neurologic signs at 40-50 days pi as a consequence of chronic demyelinating leukoencephalomyelitis (DL) in which virus persists in the CNS generating neurologic sequelae due to a discontinuous but progressive evolution of the disease (Green and Appel, 2006).
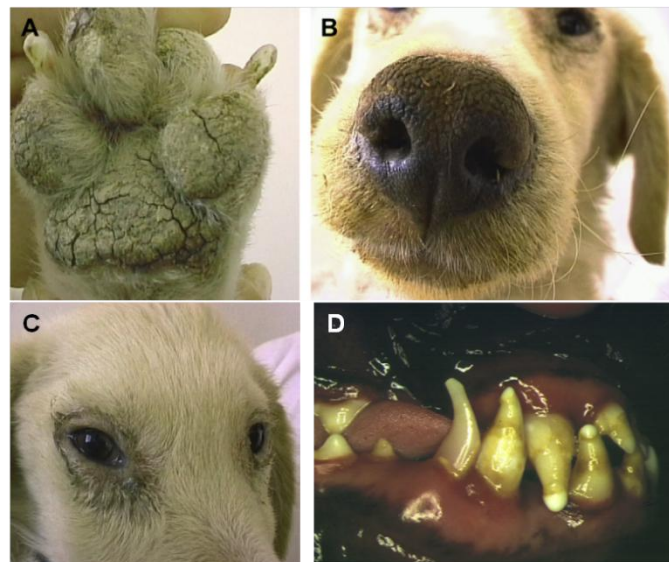
Figure 5. Dog with CDV infection. Hyperkeratosis of the foot pads (A) and nose (B). Conjunctivitis with periocular discharge (C) and marked enamel hypoplasia (D).
Adapted from: Martella V, Elia G, Buonavoglia C. Canine distemper virus.Vet Clin North Am Small Anim Pract 2008;38:787-97.

1.6 Diagnosis

Clinical diagnosis of CDV is always challenging for the clinician because of the broad spectrum of signs that can be developed by infected animals. Therefore CDV should be considered as a differential diagnosis for any febrile condition of puppies with multisystemic symptoms (Appel, 1987).

Some laboratory assays have been designed to overcome the difficulties encountered at the time of clinical diagnosis. Direct immunofluorescence test

detects viral antigen in nasal, conjucntival and vaginal smears that have been incubated with polyclonal or monoclonal anti-CDV antibodies marked with fluorescein (Hentschke, 1995). One of the disadvantages of this particularly diagnostic test is that it can confirm CDV only within 3 weeks after infection, which is the period of intensive shedding of the virus from epithelial cells; therefore it is possible to obtain false-negative results in the sub acute or chronic forms of the disease (Appel, 1987; Hentschke, 1995).

Serological assays such as the seroneutralization test, indirect immunofluorescence test and immunoperoxidase linked assay are not particularly useful for CDV laboratory confirmation, because high titers of anti-CDV antibodies may reflect prior vaccination, as well as previous clinical or subclinical infection (Frisk et al., 1999; Kim et al., 2001; Shin et al., 1995). On the other hand, when a patient is suffering from the severe form of the disease, the antibody titers may be low, due to the immunosuppressive properties of the virus (Appel, 1987). Virus-specific immunoglobulin M persists for at least 3 months after infection and may be specifically recognized by enzyme-linked immunoasorbent assay-ELISA and used as a marker of recent CDV infection (Blixenkrone-Moller et al., 1991; von Messling et al., 1999).

Definitive diagnosis can be achieve through virus isolation and identification, but this assay takes between several days to weeks; therefore, it is of limited value when applied to clinical specimens, in which a prompt diagnosis is required (Frisk et al., 1999; Kim et al., 2001; Shin et al., 1995).

Molecular techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) (Frisk et al., 1999; Kim et al., 2001; Shin et al., 1995) and Real time RT-PCR (Elia et al., 2006) have become an efficient alternative for rapid CDV diagnosis, because of their high sensitivity and specificity.

In recent years, there have been several reports of vaccinated animals infected with wild-type CDV strains that developed a severe form of the disease (Blixenkrone-Moller et al., 1993; Decaro et al., 2004; Frolich et al., 2000; Gemma et al., 1996; Scagliarini et al., 2003; Woma et al., 2010). Due to the fact

that CDV is an immunopreventable disease, and recently vaccinated animals can harbor the modified live virus in theirs tissues and secretions, some molecular essays, such as hemi nested multiplex PCR, have been developed to differentiate between field and vaccine strains. Additionally, this particularly technique uses specific probes that allow for the distinction of various CDV lineages (Martella et al., 2007).

Veterinary clinicians have widely adopted the use of the so called "rapid diagnostic tests" to confirm CDV infection, which are chromatographic immunoassays employing monoclonal anti-CDV antibodies for qualitative detection of CDV antigen in conjunctiva, urine, serum or plasma. These tests have shown maximal sensitivity and specificity (100% and 100%, respectively) when compared to nested PCR in conjunctival swabs. However, with blood lymphocytes and nasal samples, the rapid test were slightly less sensitive (89.7% and 85.7%, respectively) and specific (94.6% and 100%, respectively) than nested PCR (An et al., 2008).

1.7 Therapy and prevention

Despite great advances in canine distemper virus research, little progress has been made in therapeutic recommendations. Therapeutic approaches, although supportive and nonspecific, are beneficial because they reduce mortality. In dogs with upper respiratory infections it is advisable to keep the surrounding environment clean, warm and free of drafts. When pneumonia is complicated with secondary bacterial infections, broad spectrum antibiotics in conjunction with expectorants and nebulizations should be used (Green and Appel, 2006).

When gastrointestinal signs such as diarrhea and vomiting are present, parenteral therapy, including fluid replacement and antimicrobial therapy must be established to prevent worsening of the patient's clinical status. Supplementation with B-complex vitamins is a nonspecific therapy to replace vitamin loss due to anorexia and diuresis, and also as an effective orexigenic. Treatment with anticonvulsants should be established after the onset of systemic disease but before the development of seizures. Although there is no

evidence that shows that anticonvulsants prevent CDV entry into the CNS, this medications may suppress irritable foci from causing seizures, which may prevent seizure circuits to become established. Variable or temporary success in halting neurologic signs in some patients may result from a single anti-CNS edema dose (Green and Appel, 2006).

Currently, *in vitro* therapeutic studies for CDV have employed either synthetic inhibitors that interfere with *morbillivirus* replication (Dal Pozzo et al., 2010; Elia et al., 2008; White et al., 2007; Yoon et al., 2008), natural compounds that display anti-CDV activity *in vitro* at early and/or late stages of viral replication (Bagla et al., 2012; Gallina et al., 2011) or flavonoid compounds as promising candidates to be considered in the treatment of CDV (Carvalho et al., 2013).

The most plausible of those *in vitro* studies demonstrated that ribavirin, a purine nucleoside analogue, is capable of inhibiting CDV replication (Elia et al., 2008), but at present, these antiviral drugs are not available for veterinary use and its safety and efficacy *in vivo* have not been evaluated, therefore clinicians must use medications especially formulated for humans, a condition that can alter the clinical outcome of the veterinary patient, because little is known about the effective doses needed and possible collateral effects.

Interferon Omega is a type I interferon that has antiviral, antiproliferative, and immunomodulatory activities. *In vitro* studies demonstrated that Feline interferon omega has an antiviral activity against CDV four folds higher than that elicited by Feline interferon Alpha (Wang et al., 2008). Three clinical trials using Recombinant Feline interferon omega in patients exhibiting systemic signs of CDV infection but with no CNS clinical manifestations, showed a substantial clinical improvement in 81.6%, 92% and 81.8% of the patients, respectively (Lorenzana, 2013; Shimamura et al., 1999).

Modified live (ML) vaccines are routinely used in standard immunization protocols worldwide because they elicit long-lasting protective immunity against CDV. Some of the vaccines used at the present time include strains such as Onderstepoort, Rock born, Snyder Hill and Lederle (Appel, 1987). Several

vaccine strains may retain pathogenicity when used in wild-life species (Durchfeld et al., 1990). Immunosuppression, as well, induced either by stress or concomitant disease may result in reversion to virulence of the vaccine (Krakowka et al., 1982; Max and Appel, 1978).

To overcome passive immunity of maternal origin that interferes with vaccine efficacy in puppies, heterologous vaccination with modified live Measles virus has been employed to generate cross-reactive immunity; however this poses a risk of introducing human viruses into the environment (Appel et al., 1984; Martella et al., 2008). Some other approaches to overcome this passive immunity interference include a lipid-formulated DNA plasmid vaccine, encoding for H and F CDV membrane glycoproteins, which protects against a severe CDV virus challenge (Fischer et al., 2003).

Fischer *et al.* (2002) evaluated two canine adenovirus- 2 (CAV-2) vectored CDV vaccines expressing either CDV haemagglutinin or fusion glycoproteins in puppies born to CDV and CAV-2 vaccinated dams. CDV neutralizing antibodies were induced, and solid protective immunity against intracerebral challenge with virulent CDV was present, in all subcutaneously vaccinated pups, despite the presence of pre-existing systemic immunity to the CAV-2 vector. However, when administered intranasally to pups with pre-existing systemic immunity to CAV-2, anti-CAV-2 vector interference was shown, suggesting limitations for its use (Fischer et al., 2002; Pardo et al., 2007).

Through the use of recombinant technology, genes encoding for CDV or measles virus antigens (H and F) were inserted into the genome of vaccinia (Taylor et al., 1991) or canarypox (ALVAC®) (Stephensen et al., 1997) vector virus. Pardo *et al.* (1997) assessed immunity induced by the canarypox CDV vectored vaccine in Specific Pathogen Free beagle pups between 7 and 10 weeks old and came to the conclusion that this vaccine administered subcutaneously or intramuscular, is safe, elicits CDV seroconversion and protects vaccinated pups against virulent CDV challenge (Pardo et al., 1997).

Other authors have reported that protective humoral immunity elicited by a Commercial canarypox-vectored CDV vaccine (Recombitek®, Merial, Inc USA) can last for a minimum of 36 months when the vaccination protocol includes one initial dose and a boost of two or more doses administered approximately 4 weeks apart (Larson and Schultz, 2007). In other studies, these authors could demonstrate that one dose of a recombinant CDV (rCDV) vaccine prevented pups from dying when administered several hours before challenge and completely prevented infection in those vaccinated 7 days before infection. On the other hand, rCDV vaccine significantly enhanced (fourfold or greater) the antibody response of previously immunized adult and juvenile dogs in comparison with ML vaccines (Larson and Schultz, 2006).

Despite the development of the previously mentioned vaccination strategies, CDV ML vaccines are the main resource in immunization protocols worldwide, therefore, to overcome the interference of maternally derived antibodies, it is recommended to vaccinate puppies for the first time at 6 to 8 weeks of age and again after 4 weeks. New investigations have revealed that immunity elicited by ML vaccines persists for more than 3 years, hence vaccination of the animals is recommended every 3 years instead of annually (Day et al., 2010; Gore et al., 2005).

1.8 CDV and the Colombian perspective

Although CDV continues to be a serious problem in dogs throughout the country, little is known about the epidemiologic status of the disease in canine populations, both wild and domestic. There are several reasons that could explain this lack of information on the matter. First, in the vast majority of the cases with a presumptive CDV diagnosis, laboratory confirmation is not performed; therefore, it is difficult to establish any kind of information related to CDV prevalence in different geographic areas. Second, there is no available information regarding CDV vaccination coverage for Colombia, hence, it is impossible to estimate the percentage of dogs vulnerable to the disease, situation that poses a serious problem for the future design of massive

immunization campaigns. The third and last reason is the little development in the research field of infectious diseases of companion animals in Colombia, a condition aggravated by the lack of government support.

Despite the above mentioned situation, several teaching Hospitals from different Veterinary Medicine Faculties have made efforts to estimate the frequency of presentation of infectious diseases in their patients. In Manizales city a one year prospective study from the Veterinary Hospital of University of Caldas determined that infectious diseases were diagnosed more frequently (30.07%) and that 17.15% of these cases were confirmed as CDV (Linares et al., 2004).

An epidemiological study of CDV carried out in Medellín at University of Antioquia´s Veterinary Hospital using medical records from 1967 to 1977 found that younger animals were most affected (76%) and registered prevalence fluctuations from 1.4% to 5.1% between years 1967 and 1974 (Gómez et al., 1985). In recent years, some studies focusing on CDV laboratory confirmation techniques have been conducted at University of Antioquia´s Veterinary Hospital and several canine shelters of the city of Medellín. The percentage of dogs positive for the Direct Immunofluorescence Assay was 41.8%, and 28.2% of the patients tested positive for intracytoplasmic inclusion bodies detected by Shorr's staining (Casas et al., 2001).

The information presented so far does not reflect the real epidemiological scene for CDV in Colombia, because of the limited nature of the data in terms of population coverage. There is also a complete absence of molecular information of Colombian wild-type CDV viruses; therefore, no assumptions can be made on the type of strains circulating, if they are genetically related with strains from other South American countries or, if there is a pronounced genetic divergence with vaccine strains, as it has been reported earlier for all the wild-type strains detected so far in canine populations around the world.

In conclusion, there is an urgent need for epidemiological studies focused on disease prevalence in Colombian canine populations to guide future interventions to control CDV spread. Likewise, data concerning molecular

epidemiology of wild type strains and their circulation dynamics could elucidate the patterns of virus evolution in this specific geographic area.

2. MATERIALS AND METHODS

2.1 Clinical specimens and vaccine strains

Following an active case finding approach, a total of 46 clinical samples from dogs exhibiting signs suggestive of CDV were obtained from October 2011 through July 2012 in 12 Veterinary Hospitals and animal shelters from Aburra Valley (Antioquia Province, Colombia/South America). The inclusion criteria were nasal and/or ocular discharge, fever, respiratory, gastrointestinal and/or nervous system involvement and hyperqueratosis of footpads and nose. Basic data were at the time of sampling including age, sex, breed, vaccination status, municipality and/or neighborhood of origin, and clinical signs. Clinical specimens included serum, urine and conjunctival swabs. Four commercially available vaccines designated as Vaccine A, B, C and D were used as positive controls for RT-PCR reactions.

2.2 RNA extraction

Total RNA was extracted from 140 µl of supernatants of conjunctival swabs, serum, urine and four commercial CDV vaccines using the QIAamp Viral RNA (QIAGEN®, Hilden, Germany) Mini Spin Procedure according to manufacturer's instructions. RNA quality and quantity was determined by spectrophotometrical analysis with a Nanodrop® 1000 (Thermo Scientific, Wilmington, DE, USA), and RNA aliquots were stored at -80 ºC until use.

2.3 Complementary DNA (cDNA) synthesis

Synthesis of cDNA was performed using the RevertAid™ Premium First Strand cDNA Synthesis Kit (Thermo Scientific®, Glen Burnie, MD, USA) according to manufacturer´s instructions. Briefly, a denaturation mixture consisting of 1 µl (100 pmol/µl) of random hexamers, 1 µl of dNTPs (10 mM) and 13 µl (0.02 µg-4.6 µg) of total RNA was initially denatured at 65 °C for 5 min and immediately incubated on ice. The RT mix solution consisted of 4 µl of 5X

ReverseTranscriptase Buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl2, 50 mM DTT) and 1 µl of RevertAid™ Premium Enzyme Mix (RevertAid™ Premium Reverse Transcriptase and RiboLock™ RNase Inhibitor). The RT mixture was added to the denaturation mixture and reverse transcription was perform in a total volume of 20 µl in a Veriti™ Dx 96-Well Thermal Cycler (Applied Biosystems®, Foster City, CA, USA) for 10 min at 25 °C followed by 30 min at 50 °C; the reaction was terminated by heating at 85 °C for 5 minutes. The reaction product was stored at -80 °C until use. Ultrapure water was used as a negative control and RNA from vaccines as positive control.

2.4 PCR and sequencing

cDNAs form clinical specimens were screened by PCR amplification of the phosphoprotein gene using the Maxima Hot Start PCR Master Mix (2X) (Thermo Scientific®, Glen Burnie, MA, USA) reagent kit according to manufacturer´s instructions. Detection of viral cDNA was performed using Morbillivirus universal primers (Barret et al., 1993) (Table 1) which amplify a 429-bp fragment of the phosphoprotein gene. Four µl of cDNA were added to a PCR reaction mixture thata contained 25 µl of Maxima Hot Start PCR Master Mix (2X) (Maxima Hot Start Taq DNA polymerase 2X, Hot Start PCR buffer, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 4 mM Mg2+), 15 µl of nuclease free water and 3 µl (10µM) of each of the forward and reverse primers. PCR was performed on a Veriti™ Dx 96-Well Thermal Cycler (Applied Biosystems®) under the following conditions: an initial denaturation at 95 °C for 4 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50.82 °C for 30 seconds, extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes. Ultrapure water was used as a negative control and cDNA from one of the vaccines as positive control.

To amplify the full-length H gene, PCR was performed in all samples that tested positive for the P gene. A Maxima Hot Start PCR Master Mix (2X) (Thermo Scientific®) reagent kit was used according to manufacturer´s instructions. Detection of viral cDNA was performed using primers CDVff1 and HS2 (Lan et al., 2006) which amplify a 2100-bp fragment of the CDV genome thata

comprises the H gene and flanking regions at both ends. Four microliters of cDNA were added to a PCR reaction mixture consisting of 25 µl of Maxima Hot Start PCR Master Mix (2X) (Maxima Hot Start Taq DNA polymerase 2X, Hot Start PCR buffer, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 4 mM Mg2+), 15 µl of nuclease free water and 3 µl (10µM) of each of the primers (Table 1). PCR was performed on a Veriti™ Dx 96-Well Thermal Cycler (Applied Biosystems®) under the following conditions: initial denaturation at 95 ⁰C for 4 minutes, followed by 35 cycles of denaturation at 95 ⁰C for 30 seconds, annealing at 48.2 ⁰C for 30 seconds, extension at 72 ⁰C for 2 minutes, and a final extension at 72 ⁰C for 10 minutes.

Following PCR, 5 µl of each amplicon were analyzed by gel electrophoresis in a 1.5% agarose gel (AGAROSE I™, Amresco, Solon, OH, USA) at 110 v for 60 minutes. The gel was stained with etidium bromide and bands were visualized using a Molecular Imager® ChemiDoc TM XRS System with Bio-Rad Quantity One® software, v. 22 (Bio-Rad Laboratories, Hercules, California, USA). Product sizes were estimated using a molecular weight ladder ranging from 100 bp to 3000 bp (GeneRuler™ 100 bp Plus DNA Ladder, Thermo Scientific®).

PCR amplicons were submitted for purification and sequencing procedures to Macrogen Inc., (MacrogenInc., Seoul, Korea). Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems®), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using a set of 8 primers (Lan et al., 2006; Pardo et al., 2005) (Table 1).

Table 1. Oligonucleotides used for CDV P gene detection and for full length H gene amplification and sequencing.

Oligonucleotide label	Oligonucleotide sequence	Genomic position	Reference
	P gene Amplification	2132- 2149	Barrett *et al*, 1993
CDV Universal (forward)	ATGTTTATGATCACAGCGCGGT		Barrett *et al*, 1993
CDV Universal (Reverse)	ATTGGGTTGCACCACTTGTC	2541–2560	Barrett *et al*, 1993
	H gene amplification and sequencing		
CDVff1(Forward)	TCGAAATCCTATGTGAGATCACT	6897 – 6919	Lan *et al*., 2006
CDVHS2(reverse)	ATGCTGGAGATGGTTTAATTCAATCG	8994 – 8969	Lan *et al*., 2006
CDVHS1(Forward)	AACTTAGGGCTCAGGTAGTCC	7054 – 7074	Lan *et al*., 2006
CDVHforD (Forward)	GACACTGGCTTCCTTGTGTGTAG	7948 – 7970	Lan *et al.,* 2006
CDVHr2 (Reverse)	GTTCTTCTTGTTTCTCAGAGG	8198 – 8178	Lan *et al*., 2006
CDVP2F(Forward)	ACTTCCGCGATCTCCACT	7372 – 7389	Pardo *et al.,* 2005
CDVP3R (Reverse)	ACACTCCGTCTGAGATAGC	7760 - 7742	Pardo *et al.,* 2005
CDVP5R (Reverse)	GTGAACTGGTCTCCTCTA	8395 – 8378	Pardo *et al.,* 2005

2.5 Phylogenetic analysis of the H gene

Sequence data were assembled and edited over a total length of 1824 bp using the SeqMan program (DNAStar Lasergene software package, Madison, WI, USA). Nucleotide BLAST (Basic Local Alignment Search Tool) was used to explore the sequence similarity of Colombian CDV strains compared with all the available sequences of CDV in the NCBI nucleotide databases. Nucleotide and deduced amino acid alignments of the 15 full-length H gene sequences from dogs of the Aburrá Valley, along with 59 field isolates and vaccine strains from different geographic regions where performed in MEGA 5 (Tamura et al., 2011) using the Muscle algorithm, and and the uncorrected (p) distances for nucleotide and amino acid sequences were calculated.

The phylogenetic relationships based on the nucleotide alignment of complete H gene sequences were inferred using distance based (Neighbor Joining) and character based (Bayesian and Maximum Likelihood) approaches implemented in MEGA 5 (Tamura et al., 2011), PhyML 3.0 (Guindon et al., 2010) and

MrBayes 3.2.2 (Ronquist et al., 2012) software, respectively. The best fit model for nucleotide substitution was identified by jModeltest (Posada, 2008) as a transversional model plus gamma distribution rate heterogeneity (TVM + G) according to the Bayesian information criterion (Schwarz, 1978). The Maximum Likelihood analysis was performed using this model; however, the Bayesian inference analysis was executed with the general time-reversible model plus gamma distribution rate heterogeneity (GTR + G) because the TVM model is not implemented in MrBayes 3.2.2. For this method, two parallel analyses were run for 1000,000 generations with a 20% burn-in period. The convergence of the MCMC chains was assessed by the standard deviation of split frequencies, which fell below 0.01. America 1 lineage was used as an outgroup to root the phylogenetic trees. The consensus trees where edited in FigTree software, V.1.4 (Rambaut, 2012).

2.6 Amino acid analysis of the H protein

The deduced amino acid sequences of the H protein of Colombian wild-type CDV strains (607 aa) where aligned with cognate CDV protein sequences from different geographic regions using MEGA 5 to explore their amino acid profiles and their potential differences compared with vaccine and wild-type strains of previously sequenced CDV lineages. The prediction of potential N-linked glycosylation sites was performed with NetNGlyc 1.0 server (Gupta et al., 2004).

2.7 Description of Clinical features

A descriptive analysis of the most relevant clinical features in dogs that tested positive for RT-PCR (P gene) was made. Age, sex, breed, vaccination status, and clinical signs discriminated by affected system were considered as variables for the statistic analysis.

3. RESULTS

3.1 Detection of P gene and clinical features

A 429-bp fragment of the phosphoprotein gene (Figure 6) was detected in clinical samples from 23 (50%) of the 46 dogs sampled.
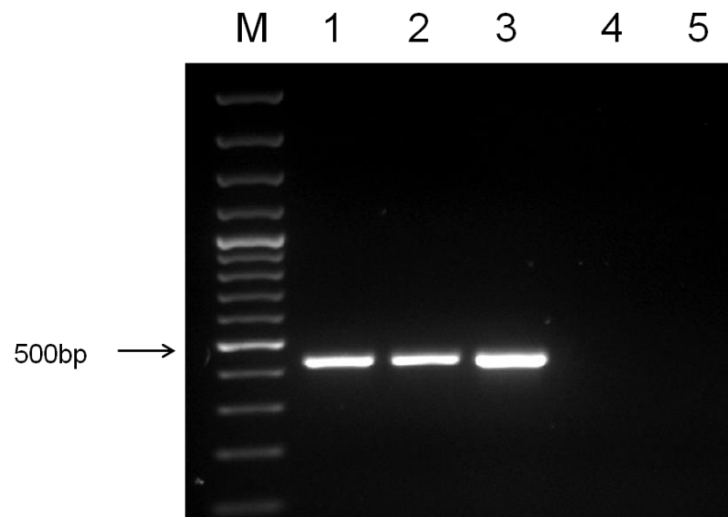
Figure 6. Agarose gel electrophoresis of the amplification products from Polimerase Chain Reaction (PCR) of the phospoprotein gene. M, Molecular weight marker; Lane 1, positive control (vaccine); Lane 2, sample 41E; Lane 3, sample 42E: Lane 4, sample 43E; Lane 5, negative control.

Fourteen (60.9%) of the dogs positive for CDV were male and nine (39.1%) were female. Animals from one to six months old were the most frequently affected (60.9%), although disease presentation in dogs older than 18 months was also observed (17.3%) (Figure 7). Regarding clinical manifestations, dual involvement of nervous and respiratory systems accounted for 34.8 % of the cases, while presentation of respiratory signs as the sole clinical manifestation was present in 30.4% of the dogs (Figure 8).
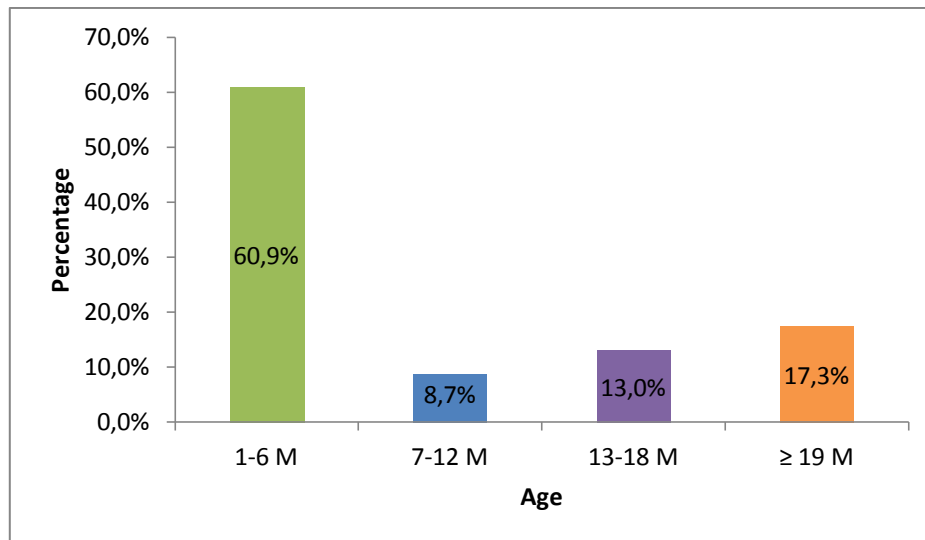
Figure 7. Frequency of CDV infection according to age intervals. M: Months.
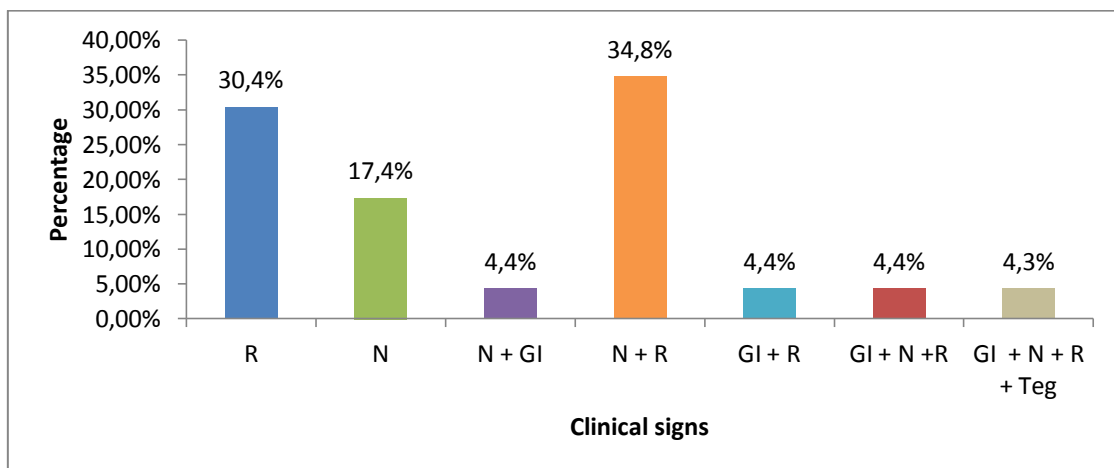
Figure 8. Frequency of CDV infection according to clinical signs. R, Respiratory; GI, Gastrointestinal; N, Neurological; T, Tegumentary.

3.2 Sequence analysis of the H gene

23 clinical samples positive for the P gene were screened by H gene amplification. A fragment of 2100-bp was amplified and sequenced (Figure 9) in 15 clinical specimens and the assembled H gene nucleotide sequences were submitted to the GenBank. Information regarding the age, gender, breed, vaccination status, clinical signs and outcome is summarized in Table 2 for the H-gene-positive samples, along with their accession numbers.
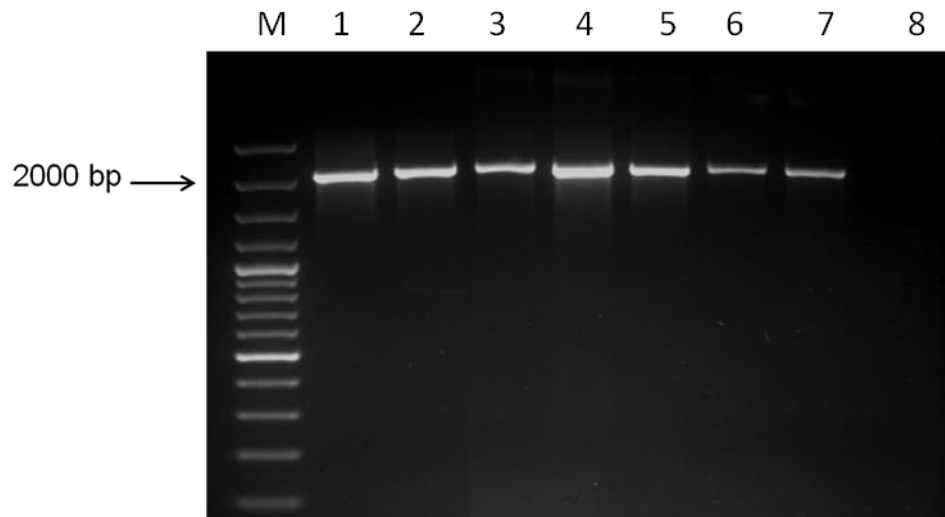
Figure 9. Agarose gel electrophoresis of the amplification products from Polymerase Chain Reaction (PCR) of the Hemagglutinin gene. M, Molecular weight marker; Lane 1, positive control (vaccine); Lane 2, sample 13E; Lane 3, sample 18E; Lane 4, sample 19E; Lane 5, sample 29E; Lane 6, sample 30E; Lane 7, sample 39E; Lane 8, negative control.

Table 2. Clinical features of Colombian dogs infected with Canine Distemper virus (CDV)

Code	Sample[a]	Sex[b]	Breed	Age[c]	Clinical signs[d]	Vaccination status	Course	GenBank Accesion Number
2-CO/11	S	F	Beagle	2M	R	Unknown	Euthanized	KF835411
13-CO/12	CS	F	Basset Hound	3M	R, N	No	Euthanized	KF835412
14-CO/12	CS	M	Pitt Bull	3M	R, N	Unknown	Euthanized	KF835413
18-CO/12	CS	M	Labrador	Unknown	R,N	Unknown	Unknown	KF835414
19-CO/12	CS	M	Mix-Breed	4M	R, N, T	Unknown	Euthanized	KF835415
26-CO/12	CS	F	Fox Terrier	4M	GI, R, N	Incomplete vaccination	Unknown	KF835416
29-CO/12	CS	F	Labrador	4 Y	N	Not Vaccinated	Euthanized	KF835417
30-CO/12	CS	F	Mix Breed	2M	R, N	Not Vaccinated	Unknown	KF835418
33-CO/12	CS	F	Mix Breed	3M	N	Not Vaccinated	Euthanized	KF835419
34-CO/12	CS	M	Pit Bull	3M	R, N	Not Vaccinated	Euthanized	KF835420
39-CO/12	CS	F	Mix Breed	1Y	R	Unknown	Recovered	KF835422
40-CO/12	CS	M	Mix Breed	5Y	R	Not Vaccinated	Recovered	KF835421
41-CO/12	CS	F	Mix Breed	8M	R, N, T	Not vaccinated	Unknown	KF835423
42-CO/12	CS	M	French Poodle	3M	GI, N	Not Vaccinated	Unknown	KF835424
44-CO/12	CS	M	Basset Hound	5M	R, N	Unknown	Unknown	KF835425

[a] S: Serum, CS: Conjuctival swabs; [b] F: female, M: male; [c] M: months, Y: Years; [d] R: Respiratory, GI: Gastrointestinal, N: Neurological, T: Tegumentary.

The H sequences of strains 19-CO/12, 2-CO/11, 41-CO/12 and 29-CO/12 were 100% identical to each other. Similarly, the sequences for 42-CO/12 and 13-CO/12 were 100% identical, as were the sequences for strains 40-CO/12 and 30-CO/12. Therefore, only sequences 2-CO/11, 13-CO/12 and 40-CO/12 where included with the rest of the Colombian strains in the phylogenetic analysis. The vaccines used as positive controls in the PCR reactions were also sequenced.

A preliminary search in BLASTn revealed that the Colombian wild-type viruses showed a 97% maximum nucleotide identity to vaccine strain Rockborn-Candur (GenBank accession number GU266280) and 96% nucleotide identity to a lesser panda isolate from china (AF178039), as well as a dog (JN008903) and a fox (JN008906) isolates from Greece. Field viruses from Colombia displayed low identity with vaccine strains belonging to the America 1 lineage (91.3-91.8% nt and 89.6-91.2% aa).

Blast searches performed with sequence data from the commercial vaccines used as positive controls in this study revealed that Vaccine A had a 99% percent identity with the Ondeersteport Strain (AF378705) and Vaccines B and C showed 100% percent identity with the Lederle vaccine strain (DQ903854). In addition, Vaccine D displayed 99% percent identity with a Rockborn strain (GU266280) and a lesser panda isolate (AF178039).

The phylogenetic relationships based on the nucleotide alignment of complete H gene sequences inferred by distance (Neighbor Joining) and character approaches (Maximum likelihood and Bayesian inference) resulted in trees with similar topology. Our analysis identified 11 geographical lineages, 10 of them which had been previously reported (Blixenkrone-Møller et al., 1992; Haas et al., 1997; Harder et al., 1996; Harder et al., 1993; Iwatsuki et al., 2000; Orvell et al., 1990; Woma et al., 2010). A novel lineage comprising all the Colombian wild-type viruses formed a monophyletic group with Bayesian posterior probabilities and bootstrap values (Maximum likelihood and p-distance) consistently high (Figures 10-12).

In the phylogenetic tree inferred by the Neighbor-Joining method (Figure 10) the bootstrap support values for each of the lineages were above 96%, with the exception of European Wildlife and South America 2 lineages that clustered together in the phylogenetic tree with a bootstrap value of 52%. In the phylogram infered by Maximum Likelihood (Figure 11) the bootstrap support values for each of the lineages were above 97.2%, with the exception of European Wildlife and South America 2 lineages that clustered together in the phylogenetic tree with a bootstrap support value of 80.5%. In the phylogenetic tree inferred by the Bayesian method and rooted with America 1 lineage (Figure 12). The Bayesian posterior probability value for each of the lineages was 1. South America 2 lineage clustered together with European Wildlife lineage in the phylogenetic tree, with a Bayesian posterior probability value of 1.

Wild type strains from Aburrá Valley clustered together in all the phylogenetic trees and they appeared immersed in a super clade containing America 2, European Wildlife, Europe/south America 1, South America 2, Rock born Like and Asia 1 lineages.
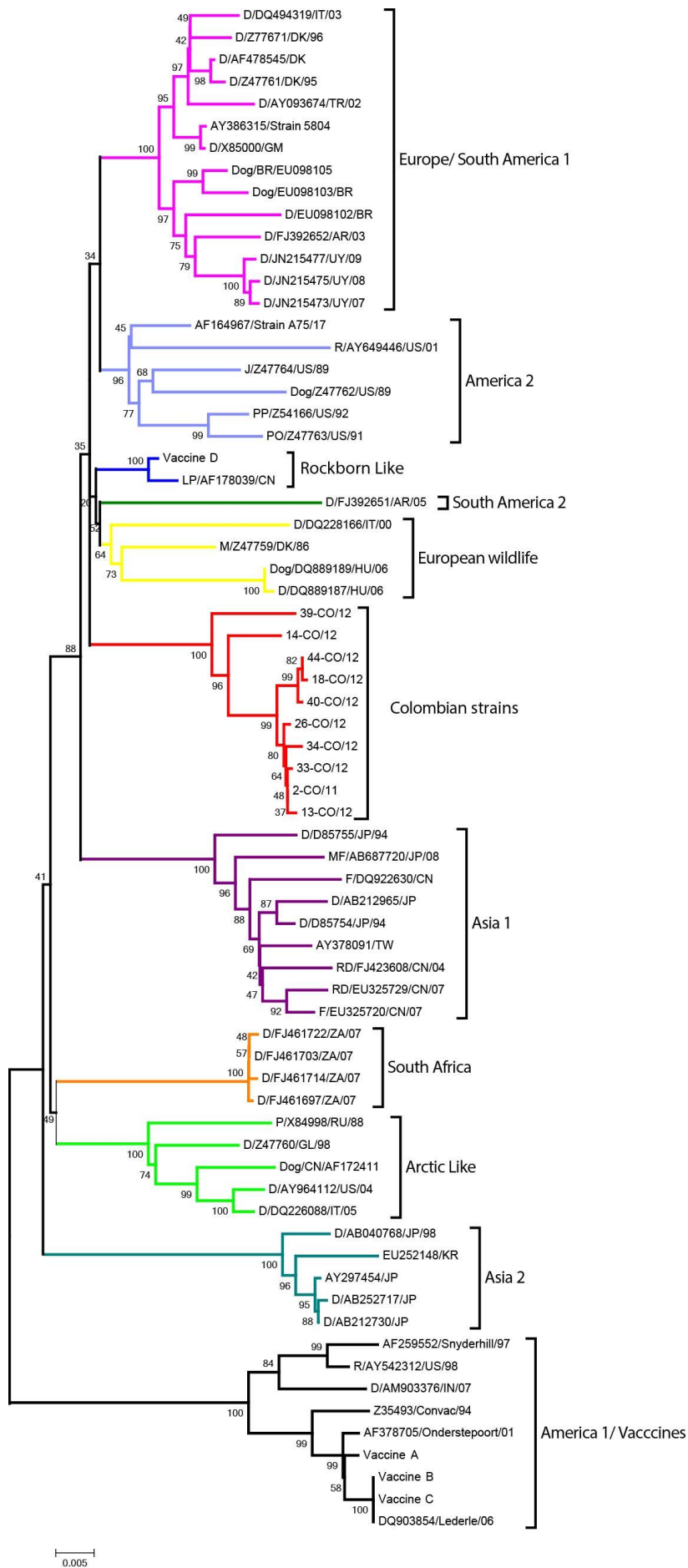
Figure 10. Phylogenetic relationships between 69 CDV strains based on H gene sequences. The phylogenetic tree was inferred by the Neighbor-Joining method using 1000 replicates. The species from which each isolate was obtained, GenBank accession numbers, country of origin and year of isolation are indicated in the tip labels if available. Numbers at the nodes are bootstrap values for the clade. Abbreviations for animal species: D= Dog (*Canis familiaris)*, F= Fox (*Vulpes vulpes*), J= Peccary *(Pecari tajacu)*, LP= Lesser panda (*Ailurus fulgens*), M= Mink (*Mustela lutreola*), MF= Crab-eating macaque (*Macaca fascicularis*), P=Baikal seal (*Phoca sibirica),* BL= Black leopard (*Panthera onca*), R= Racoon (*Procyon lotor*), RD= Racoon dog (*Nyctereutes procyonoides*). Abbreviations for country: AR= Argentina, BR= Brasil, CN= China, CO= Colombia, DK= Denmark, GL= Green Land, GM=Germany, HU= Hungary, IN= India, IT= Italy, JP= Japan, KR= South Korea, RU= Russia, TR= Turkey, TW= Taiwan, US= United States, UY= Uruguay, ZA= South Africa.

Z35493/Convac/94
AF378705/Ondeersteport/01
Vaccine A
DQ903854/Lederde/06
Vaccine C
Vaccine B
America 1/ Vaccines

D/AM903376/IN/07
AF259552/Snyderhill/97
R/AY542312/US/98

D/D87755/JP/94
MF/AB687720/JP/08
F/DQ922630/CN
D/AB212965/JP
D/D85754/JP/94
AY378091/TW
RD/FJ423608/CN/04
RD/EU325729/CN/07
F/EU325720/CN07
Asia 1

39-CO/12
14-CO/12
40-CO/12
44-CO/12
18-CO/12
26-CO/12
34-CO/12
33-CO/12
2-CO/11
13-CO/12
Colombian Strains

D/EU098105/BR
D/EU098103/BR
D/JN215477/UY/09
D/JN215475/UY/08
D/JN/215473/UY/07
D/FJ392652/AR/03
D/EU098102/BR
AY386315/ Strain 5804
D/X85000/GM
D/AY093674/TR/02
D/AF478545/DK
D/Z477617DK795
D/DQ494319/IT/03
D/Z77671/DK/06
Europe / South America 1

PP/Z54166/US/92
PO/Z47763/US/91
AF164967/ Strain A75/17
R/AY649446/US/01
J/Z47764/US/89
D/Z47762/US/89
America 2

Vaccine D
LP/AF178039/CN
Rockborn Like

D/FJ392651/AR/05
South America 2

D/dq228166/IT/00
M/Z47759/DK/86
D/DQ889189/HU/06
D/DQ889187/HU/06
European Wildlife

D/FJ461697/ZA/07
D/FJ461703/ZA/07
D/FJ461722/ZA/07
D/FJ461714/ZA/07
Africa

D/AB040768/JP/98
EU252148/KR
AY297454/JP
D/AB252717/JP
D/AB212730/JP
Asia 2

P/X84998/RU/88
D/Z47760/GL/98
D/CN/AF172411
D/AY964112/US/04
D/DQ226088/IT/05
Arctic Like

Figure 11. Phylogenetic relationships between 69 CDV strains based on H gene sequences. The phylogenetic tree was inferred by the Maximum Likelihood method using 1000 replicates. The species from which each isolate was obtained, GenBank accession numbers, country of origin and year of isolation are indicated in the tip labels if available. Numbers at the nodes are Bootstrap values for the clade. Abbreviations for animal species: D= Dog (*Canis familiaris)*, F= Fox (*Vulpes vulpes*), J= Peccary *(Pecari tajacu)*, LP= Lesser panda (*Ailurus fulgens*), M= Mink (*Mustela lutreola*), MF= Crab-eating macaque (*Macaca fascicularis*), P=Baikal seal (*Phoca sibirica),* BL= Black leopard (*Panthera onca*), R= Racoon (*Procyon lotor*), RD= Racoon dog (*Nyctereutes procyonoides*). Abbreviations for country: AR= Argentina, BR= Brasil, CN= China, CO= Colombia, DK= Denmark, GL= Green Land, GM=Germany, HU= Hungary, IN= India, IT= Italy, JP= Japan, KR= South Korea, RU= Russia, TR= Turkey, TW= Taiwan, US= United States, UY= Uruguay, ZA= South Africa.

Figure 12. Phylogenetic relationships between 69 CDV strains based on H gene sequences. The phylogenetic tree was inferred by Bayesian analysis with 1000,000 generations. The species from which each isolate was obtained, GenBank accession numbers, country of origin and year of isolation are indicated in the tip labels if available. Numbers at the nodes are posterior probabilities for the clade. Abbreviations for animal species: D= Dog (*Canis familiaris)*, F= Fox (*Vulpes vulpes*), J= Peccary *(Pecari tajacu)*, LP= Lesser panda (*Ailurus fulgens*), M= Mink (*Mustela lutreola*), MF= Crab-eating macaque (*Macaca fascicularis*), P=Baikal seal (*Phoca sibirica)*, BL= Black leopard (*Panthera onca*), R= Racoon (*Procyon lotor*), RD= Racoon dog (*Nyctereutes procyonoides*). Abbreviations for country: AR= Argentina, BR= Brasil, CN= China, CO= Colombia, DK= Denmark, GL= Green Land, GM=Germany, HU= Hungary, IN= India, IT= Italy, JP= Japan, KR= South Korea, RU= Russia, TR= Turkey, TW= Taiwan, US= United States, UY= Uruguay, ZA= South Africa.

The ten Colombian wild-type strains that were analyzed displayed high identities to each other (97.7-99.9% nt; 97.5-100% aa); however, strains 14-CO/12 and 39-CO/12 had a variation >1.5% nt (>1% aa) and >2.1% nt (>1.8% aa) respectively, from the principal cluster, which was comprised of isolates 40-CO/12, 44-CO/12, 18-CO/12, 34-CO/12, 33-CO/12, 2-CO/11, 26-CO/12 and 13-CO/12, that exhibited high similarity between each other (99.3-99.9% nt; 99-100% aa). The overall mean distance was 0.055 (SE, 0.003) and Colombian wild-type viruses showed an amino acid variation ≥ 5% from the already reported CDV lineages (Table 3).

Table 3. Uncorrected (p) distances observed over amino acid sequence pairs between CDV lineages.

	EU/SA 1	Rck L	AM 1	A 1	AM 2	A 2	Arc L	EWL	Africa	SA 2	SA3
EU/SA 1		0,006	0,009	0,007	0,005	0,009	0,007	0,006	0,008	0,007	0,007
Rck L	0,040		0,009	0,007	0,006	0,009	0,008	0,006	0,008	0,008	0,008
AM 1	0,086	0,086		0,010	0,009	0,011	0,010	0,009	0,010	0,011	0,010
A 1	0,053	0,048	0,095		0,007	0,009	0,008	0,007	0,008	0,009	0,008
AM 2	0,041	0,040	0,087	0,053		0,009	0,007	0,005	0,007	0,007	0,007
A 2	0,071	0,068	0,103	0,072	0,073		0,009	0,009	0,009	0,010	0,010
Arc L	0,058	0,054	0,091	0,067	0,058	0,075		0,007	0,008	0,009	0,009
EWL	0,048	0,041	0,086	0,056	0,047	0,071	0,058		0,007	0,007	0,007
Africa	0,052	0,049	0,089	0,056	0,053	0,067	0,056	0,052		0,009	0,008
SA 2	0,045	0,048	0,094	0,065	0,053	0,079	0,068	0,053	0,062		0,009
SA 3	0,050	0,050	0,098	0,065	0,053	0,080	0,070	0,056	0,056	0,061	

EU/SA 1: Europe/South America 1; Rck L: Rockborn Like; AM 1: America 1; A 1: Asia 1; AM 2: America 2; A 2: Asia 2; Arc L: Arctic Like; EWL: European Wild Life; SA 2: South America 2; SA 3: South America 3 (All the Colombian strains are comprised in SA 3 lineage). Standard error estimates are shown above the diagonal (in blue) and were obtained by a bootstrap procedure of 1000 replicates.

3.3 Amino acid analysis of the H protein

The deduced amino acid sequences of Colombian wild-type viruses resulted in a full-length H protein of 607 amino acids. The alignment of Colombian CDVs with isolates from different geographic regions along with vaccine strains, showed that Colombian CDVs have a unique amino acid profile with specific substitutions: T213A, N261S, F353L, Y427H, and G488R. Interestingly, all of the Colombian wild-type viruses, except the 14-CO/12 and 39-CO/12 strains had the additional substitutions I345V, I522T, T544S and E582K, which are also unique for Colombian CDVs (Figure 13).

Colombian strains showed the same residue of isoleucine at position 506 as vaccine strains belonging to America 1 lineage. Several substitutions in the Colombian strains were also present in other wild-type strains from different lineages: K22R (America 2), V41I (European Wildlife), N128S (Africa), K281R (America 2), G314S (Asia 2 and America 2), I315V (European Wildlife) and P443S (Europe/SA 1). (Figure 13). All Colombian strains had an asparagine at position 530, which is also present in wild and domestic carnivores from the Arctic-like, Africa and America 1 lineages.

Colombian wild-type viruses have 8 potential glycosylation sites (N-X-S/T) at positions 19-21, 149-151, 309-311, 391-393, 422-424, 456-458, 587-589, 603-605 (Figure 13) that are common to other lineages. The potential glycosylation site present at position 584-586 of the Asia 1 lineage, was not present in the local strains (Mochizuki et al., 1999).

```
          1         10        20        30        40        50        60        70        80        90       100       110       120
Consensus MLSYQDKVGAFYKDNARANSSKLSLVTEEQGGRRPPYLLFVLLILLVGIMALLAITGVRFHQVSTSNMEFSRLLKEDMEKSEAVHHQVIDVLTPLFKIIGDEIGLRLPQKLNEIFQFIIQKT
OP-V      ..............T.........H..................L...............................................................................
Sny-V     ...........P............H.............L...S......K..........................................................................
Led-V     ..P.................T....GH...........L..............I......................................................................
Rock-V    ...........................................................................................................................
2-CO/11   .................R..................I...................................................................V...................
13-CO/12  .................R..................I...................................................................V...................
14-CO/12  ...................................I...................................................................V...................
18-CO/12  .................R..................I...................................................................V...................
26-CO/12  .................R..................I...................................................................V...................
33-CO/12  .................R..................I...................................................................V...................
34-CO/12  .................R..................I...................................................................V...Q...............
39-CO/12  .............................V.....I...................................................................V...................
40-CO/12  .................R..................I...................................................................V...................
44-CO/12  .................R..................I...................................................................V...................
AM 2      .................R.......D.E..............I.......................I.....................................V...................
EU/SA 1   .......................................T................................................................V...................
SA 2      .......................................T........................................M.....................V...................
EWL°      ......................S....................................................................................................
EWL       .......S................H.S.....I..................A........................................................................
ArcL      .........L.................................................................D...............................................
A 1       .................................F..T..I..L................................................................................
A 2       ....................................L....A....R..V..G..D.L....M............................................................
ZA        ............................I.........VL....................................................................................

          130       140       150       160       170       180       190       200       210       220       230       240
Consensus NFFNPXREFDFRDLHWCINPPSKIKVNFTNYCDTIGIRKSIASAANPILLSALSGGRGDIFPPYRCSGATTSVGRVFPLSVSLSMSLISRTSEIINMLTAISDGVYGKTYLLVPDYIEGEFD
OP-V      ....N.................V.......ES....A...............S.....H................K...............................D..R...
Sny-V     ....N.................V.......E.....................S.....H................K...............V...............D..R...
Led-V     ....N.................V.......ES....A...............S.....H................K...............V...............D..R...
Rock-V    ....N................L......L..............R.............F..................................................
2-CO/11   ....S.....................................................................................A.........................
13-CO/12  ....S.....................................................................................A.........................
14-CO/12  ....S.....................................................................................A.........................
18-CO/12  ....S.....................................................................................A.........................
26-CO/12  ....S.....................................................................................A.........................
33-CO/12  ....S.....................................................................................A.........................
34-CO/12  ....S.....................................................................................A.........................
39-CO/12  ....S..........................V..........................................................A.........................
40-CO/12  ....S.....................................................................................A.........................
44-CO/12  ....S.....................................................................................A.........................
AM 2      ....N.................T.....................S...........................................S.........................V
EU/SA 1   ....N.............................V.........S.............................K...T...........
SA 2      ....N.............S....................N..A..I.............A............D...............E
EWL°      .......................................S.........A.................................................
EWL       ....K....................L.......R.......A.........................................................
ArcL      ....N................L.........S.............KS.......A.....S....................................
A 1       ....N...........V.VK...T.....I.....A................................................
A 2       ....N......N...V.E..............K.....................................
ZA        ....S...........S.....K.....K.................M.........................R.....

          250       260       270       280       290       300       310       320       330       340       350       360
Consensus TQKIRVFEIGFIKRWLNDMPLLQTTNYMVLPENSKAKVCTIAVGELTLASLCVDESTVLLYHDSNGSQDSILVVTLGIFGATPMDQVEEVIPVAHPSVEKIHITNHRGFIKDSIATWMVPAL
OP-V      ..E...........................................E.......S...G......W....HI....M...
Sny-V     ..E...........................................E.......R...G....S..HI....M...
Led-V     ..E...........................................E.......S...G....W...HI....M...
Rock-V    ..........................................................G...
2-CO/11   ...................S..................R...................V.....I.......V....L..
13-CO/12  ...................S..................R...................V.....I.......V....L..
14-CO/12  ...................G..................R...................V.....I.......V....L..
18-CO/12  ...................S..................R...................V.....I.......V....L..
26-CO/12  ...................S..................R...................V.....I.......V....L..
33-CO/12  ...................S..................R...................V.....I.......V....L..
34-CO/12  ...................S..................R...................V.....I.......V....L..
39-CO/12  ...................S................G.....................V...........L..
40-CO/12  ...................S..................R...................V.....I.......V....L..
44-CO/12  ...................S..................R...................V.....I.......L..
AM 2      .....................F..A........R...................
EU/SA 1   ...............N.......................G......
SA 2      ..................S.........................WG........K.I...
EWL°      ...................P..................G.......GV......
EWL       ...........................................G...
ArcL      ..............F...................A....G....G....................V...
A 1       S...........................T..............NG.......S....I...R....V...V.
A 2       ..................................................N....I...I...V...
ZA        .....................D..........G....

          370       380       390       400       410       420       430       440       450       460       470       480
Consensus VSEKQEEQKNCLESAQQRKSYPMCNQTSWEPFGGGQLPSYGRLTLPLDPSIDLQLNISFTYGPVILNGDGMDYYESPLLDSGWLTIPPKNGTVIGLINKASRGDQFTVIPHVLTFAPRESSG
OP-V      A.......G........T...........R......A.V.............N......IV....G.....L.....W...
Sny-V     A.......G........T...........R......A.V...........V...N......N..........G.
Led-V     A.......G........T...........R......A.V.............N......IF....G.....L...
Rock-V    ...........................................................................R
2-CO/11   .................................H...........S.S.........S....R
13-CO/12  .................................H...........S.S.............R
14-CO/12  .................................H...........S.S.............R
18-CO/12  ...............K...E.............H...........S.S.............R
26-CO/12  .................................H...........S.S.............R
33-CO/12  .................................H...........S.S.............R
34-CO/12  .................................H...........S.S.............R
39-CO/12  .................................H...........S..............R
40-CO/12  ...................E.............H...........S.S.............R
44-CO/12  ...................E.............H...........S.S.............R
AM 2      ................I.........................N.....E...G...........T...
EU/SA 1   ...............T..............S............G.S.S....
SA 2      ................................................M...........G.
EWL°      ...........................G...................R...T...
EWL       ..N.......................I........I...........T...
ArcL      ..NL...........A..R.......I.........S....
A 1       ....................................AT...
A 2       .Q..G.......E..........V...A.......N.F.......I...T...
ZA        .R....I..........................A...............S...

          490       500       510       520       530       540       550       560       570       580       590       600   607
Consensus NCYLPIQTSQIMDKDVLIESNLVVLPTQNFRYVIATYDISRNDHAIVYYVYDPIRTISYTYPFRLTTKGRPDFLRIECFVWDDDLWCHQFYRFEADITNSTTSVENLVRIRFSCNRSKP
OP-V      .........I.R.....I......S...........S.............H...............N.........A...........*N.
Sny-V     .........I.R.....I......S...........F..F.............N.....Y.N.A.............N.
Led-V     .........I.R.....I......S...........S.............H...............N..........A...........*N.
Rock-V    ......F.......T..........D.....F.................F...
2-CO/11   ................................T.............S.............K.........
13-CO/12  ................................T.............S.............K.........
14-CO/12  .........................................................................
18-CO/12  ................................T.............S.............K.........
26-CO/12  ................................T.............S.............K.........
33-CO/12  ................................T.............S....PH.......K.........
34-CO/12  ...............R................T.............S.............K.........
39-CO/12  .........................................................................
40-CO/12  ................................T.............S.............K.........
44-CO/12  ................................T.............S.............K.........
AM 2      .......G......T....G...........R..V.......H................A.......GS....
EU/SA 1   ...........T.................G.......................................D....
SA 2      ...........A...........D.............
EWL°      ....R...T...........N...D...
EWL       ...........T...........D...........Y.....
ArcL      ........................................
A 1       ...........T...........G...............N...
A 2       ...............T.....V.....EN...........S....D........
ZA        S................T....A.......................T....D.........
```
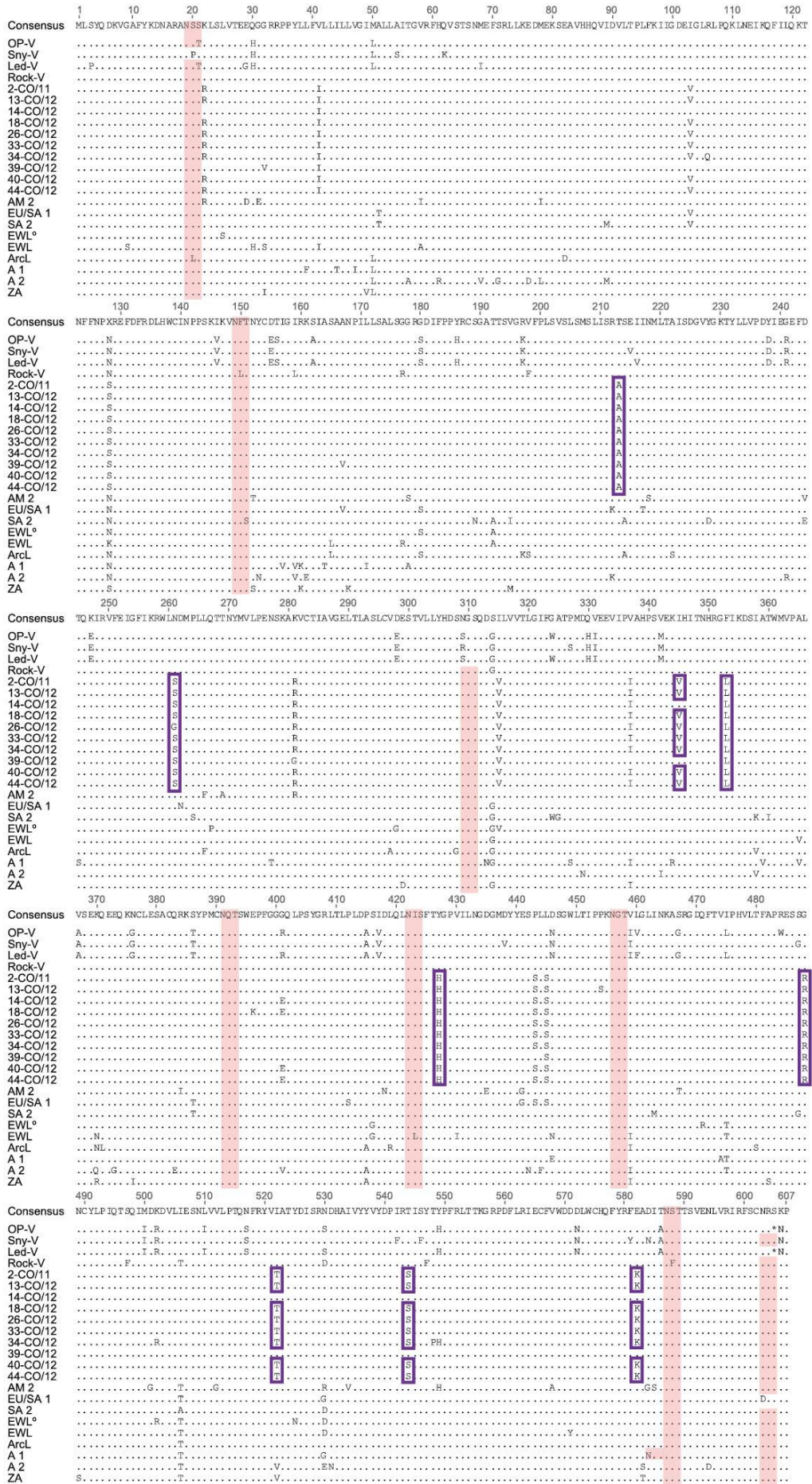
Figure 13. Alignment of the deduced amino acid sequences for the H protein of wild-type and vaccine strains of CDV. Abbreviations for the sequences: OP-V (Ondeersteport vaccine, AF378705), Led-V (Lederle vaccine, DQ903854), Sny-V (Snyder Hill vaccine, AF259552), Rock-V (Rockborn vaccine, GU266280), CO/13-39 (Colombian wild-type CDV strains), AM 2 (America 2, AY649446), EWL (European Wildlife, DQ889187), EWLº (European Wildlife, Z47759), ZA (Africa, FJ461703), EU/SA1 (Europe/ South America 1, Z47761), SA 2 (South America 2, FJ392651), ArcL (Arctic Like, Z47760), A2 (Asia 2, AB040768), A1 (Asia 1, FJ423608). Amino acids unique to Colombian isolates are boxed. Potential N-linked glycosylation sites (N-X-S/T) are shaded in pink.

4. DISCUSSION

In this study we provide the first genetic evidence of CDV in Colombia. This was achieved by the development of an RT-PCR technique to confirm CDV infection in different clinical samples, including serum, urine and conjunctival swabs. Although this technique has shown to be highly sensitive and specific (Frisk et al., 1999), it does not offer a good alternative to the widely used chromatographic immunoassay because it takes longer to execute (approximately 1 day), and early CDV detection is essential to establish appropriate treatment and quarantine measures (An et al., 2008). However, RT-PCR could be very useful and even complement the chromatographic immunoassay in sub-acute or chronic stages of the disease where there is poor shedding of the virus and a highly sensitive technique is needed to confirm the diagnosis, and also because it can be implemented in other clinical samples as cerebrospinal fluid, and tissue (Frisk et al., 1999).

The descriptive analysis of basic data gathered from clinical records of the dogs that tested positive for CDV (RT-PCR of the P gene) showed that animals from one to six months of age were most affected. This correlates with the loss of maternal antibodies between 8-12 weeks of age, as well as the absence of vaccination in dogs older than 12 weeks of age, or even errors in the immunization schedule (Day et al., 2010). Most of the dogs that tested positive for RT-PCR were unvaccinated, including the adult ones. It has been estimated that only 30-50% of the pet animal population is vaccinated, and this is significantly less in developing nations (Day et al., 2010). This explains why CDV is highly prevalent in South American countries, including Colombia.

Concomitant presentation of nervous and respiratory signs or the presence of respiratory signs as the sole clinical feature was observed more frequently in the patients included in this study (65.2%); however, a little percentage of the dogs showed gastrointestinal and dermatologic signs also. These observations reflect the broad spectrum of clinical signs documented for CDV, which correlate with virus dissemination to epithelial cells of different organs

as well as CNS (Green and Appel, 2006). In this context, it has been documented that at day 10 pi dogs exhibit the catarrhal form of the disease (Conjunctivitis, respiratory and gastrointestinal signs), and that at approximately day 20 pi the central nervous form (Myoclonus, ataxia, disturbances in consciousness) is evident (Blixenkrone-Moller et al., 1993; Green and Appel, 2006); therefore, the clinical signs observed most frequently in the dogs assessed in this study could correlate with the stage of viral infection; however, three dogs (29-CO/12, 32-CO/12 and 33-CO/12) showed CNS signs exclusively, with no previous catarrhal form of the disease. This could be due to a highly neurovirulent strain that although being genetically similar (Figures 10-12) to the other wildtype strains recovered from the Aburrá Valley (sequence data was available only for animals 29-CO/12 and 33-CO/12), could have different biological properties (Appel et al., 1994). Other reason for the presentation of CNS signs with no evident catarrhal form of the disease is the presence of intermediate levels of cell-mediated immunoresponsiveness and delayed antibody titers by days 9 to 14 pi in these animals, enabling virus persistence in their Central Nervous System (Green and Appel, 2006; Summers et al., 1984).

Since the early 90's, phylogenetic studies attempting to characterize the existing diversity of wild-type CDV strains have identified so far ten different lineages in carnivore populations around the world (Blixenkrone-Møller et al., 1992; Haas et al., 1997; Harder et al., 1996; Harder et al., 1993; Iwatsuki et al., 2000; Orvell et al., 1990; Woma et al., 2010). In this study we could demonstrate that the CDV samples analyzed grouped into a distinct monophyletic cluster in the phylogenetic tree, clearly separated from the already known wild-type and vaccine lineages (Figures 10-12). On the other hand, our CDV strains showed a high degree of amino acid identity between each other (>97.5) and a level of amino acid variation ≥ 5 with other lineages dispersed around the world (Table 3). Based on the criteria for lineage identification (Bolt et al., 1997; Martella et al., 2006; Mochizuki et al., 1999) where lineages have within-group amino acid diversity of less than 3.5% and between-group amino acid diversity greater than 4%, Colombian CDV strains should be considered a new lineage. In this context, we propose naming this

new lineage "South America 3". Due to the limitations in sample size (15 sequences) and that it was not possible to obtain clinical samples from dogs in other regions of Colombia, it remains uncertain if the same lineage currently circulates in other cities of the country.

This information suggests that there are at least three CDV lineages circulating in South America. One of them, previously reported in European countries, is prevalent in Uruguay and Brazil (Europe/ South America 1) (Budaszewski Rda et al., 2014; Panzera et al., 2011); the second one, detected in Argentinean wild and domestic carnivores (Calderon et al., 2007; Panzera et al., 2011), although showing sufficient amino acid divergence to be considered a new lineage (Table 3), is clustered within European Wildlife lineage, with Bayesian posterior probabilities and Maximum likelihood bootstrap values of 1 and 80.5%, respectively (Figures 11 and 12); however, in the Neighbor joining tree, determined with the p-distance method, the bootstrap support for this lineage was 52% (Figure 10). This suggests that South America 2 lineage is somehow related to European CDV strains isolated from wildlife. Finally, a third lineage, circulating in Antioquia-Colombia, appears to be genetically unrelated to the other lineages encountered in other parts of the world, including the previously reported South American CDV strains. The origin of this novel lineage cannot be inferred at this point since the clade emerges from the same polytomy as other lineages in the phylogenetic tree (Figures 10-12). It remains unknown whether CDV strains, genetically similar to those reported here circulate in other regions of northern South America. This hypothesis should be confirmed by extending phylogenetic studies to other cities of Colombia and neighboring countries.

Although slight differences were observed in tree topology and branch support values (Figures 10-12) according to the method used (NJ, ML and Bayesian) to infer them, the phylogenetic trees were very similar. The topological differences observed between the trees could be attributed to the different assumptions inherent to each method. Also, it has to be taken into account that the Neighbor Joining tree was generated using p distance, while ML and Bayesian trees were constructed using two different models of nucleotide

substitution (TVM + G and GTR + G, respectively), due to programming restrictions in Mr. Bayes. However, wildtype strains from Aburrá Valley clustered together in all the phylogenetic trees with high branch supports and they also appeared immersed in a super clade containing America 2, European Wildlife, Europe/south America 1, South America 2, Rock born Like and Asia 1 lineages. It is also worth noting that no strain was grouped in a different lineage depending on the method used to infer each of the phylogenetic trees.

A high degree of amino acid identity was present among the Colombian strains (97.5-100% aa), although the strains 14-CO/12 and 39-CO/12 showed higher variation at the amino acid level compared with the other Colombian strains (>1% aa and >1.8% aa, respectively) and were therefore separated from the principal cluster but remained within the monophyletic clade. The high similarity among the principal cluster sequences could be attributed to the short period of sampling and the proximity of the populations tested. Interestingly, strain 39-CO/12 came from a female dog in a shelter affected by a CDV outbreak and located 22 km from Medellin, the city where most of the other samples were obtained. This seems to suggest that the geographic pattern of CDV diversity is also present at the regional level.

Colombian CDV strains showed an amino acid variation above 9.8% with vaccine strains of the America 1 lineage and 5% with Rockborn Like lineage. The blast search performed with sequence data from the commercially available vaccines used as positive controls showed that three vaccines (A, B and C) contained Ondeersteport and Lederle strains, which was consistent with the information specified by the manufacturer. These strains were isolated approximately 50 years ago and are actively used in immunoprophylactic programs worldwide (Pardo et al., 2007; Taguchi et al., 2012). Interestingly, vaccine D, for which there was no available CDV strain information on the label, displayed 99% identity with the 46th laboratory passage of the vaccine strain Rockborn and also 99% identity with an isolate from a Lesser panda in China. The Rockborn vaccine was produced in the 1950´s through the

attenuation of a field virus in primary dog kidney cells (Rockborn, 1958), but since the early 1980´s, reports of residual virulence in vaccines containing the Rockborn strain (Cornwell et al., 1988) led to its withdrawing from several markets (Gloyd, 1995) because it was considered to be nor as safe as its counterpart, Ondeersteport strain.

Surprisingly, recent findings from Martella et al. (2011) showed that some field CDVs isolated from both domestic and wild carnivores displayed nucleotide identities above 99% with the Rockborn strain; therefore, they are considered as Rockborn-like viruses. A phylogenetic analysis showed that these viruses were genetically related to viruses from America 2 lineage and distantly related to America 1 lineage, which comprises most of the vaccines. These findings could suggest residual virulence of vaccines, circulation of Rockborn-like viruses in the field or that animalized vaccine-derived viruses were introduced in unrelated geographical settings on several occasions and circulated in the area in different carnivores, taking advantage of the broad host range of CDV (Martella et al., 2011). Field strains circulating in Colombian dogs displayed 97% nucleotide identity to Rockborn strain and an amino acid variation of 5%, therefore they should not be considered as Rockborn-like viruses; however, the use of vaccines with the Rockborn strain in canine populations could increase the incidence of residual virulence or elicit the circulation of Rockborn–like viruses in this geographical area in a distant future.

The pronounced genetic diversity reported in CDV isolates around the world has raised concerns over a possible alteration in the antigenic profile of these new variants that could compromise the immunity elicited by vaccine strains (Blixenkrone-Moller et al., 1993; Ek-Kommonen et al., 1997; Iwatsuki et al., 2000; Martella et al., 2006; Martella et al., 2008; Uema et al., 2005). CDV infection was confirmed in four vaccinated animals in this study (21-CO/12, 22-CO/12, 23-CO/12 and 26-CO/12); however, only one full-length H sequence was obtained, which came from the animal 26-CO/12. The phylogenetic analysis (Figure 12) rules out any possibility of disease caused by a vaccine strain with residual virulence; therefore, the most plausible explanation for

CDV infection in the four-month-old puppy that had received only one vaccination, is failure to mount a primary immune response due to an inappropriate vaccination schedule. Common additional reasons for vaccine failure include the presence of maternally derived antibodies and inappropriate handling or administration of vaccines (Appel et al., 1981; Blixenkrone-Moller et al., 1993). It was interesting that the other three dogs positive for CDV (21-CO/12, 22-CO/12 and 23-CO/12) had previous vaccination records and where older than 12 months. This could indicate that the animals were infected by a wildtype strain that could escape the immune response elicited by vaccines, an inappropriate vaccination or immunodeficiency. However, no assumptions can be made because there is no sequence data available from this animals.

Extensive pathogenesis studies have revealed that CDV initially infects lymphocytes and massively replicates in them, causing the classical immunosuppression seen in all *morbilliviruses*; afterwards, epithelial cells become infected (von Messling et al., 2004) and neurologic dissemination follows in most of the cases (Beineke et al., 2009). Hemagglutinin protein plays an essential role in viral tropism as it interacts with the SLAM, also known as the universal *Morbillivirus* receptor (Erlenhoefer et al., 2001; Tatsuo et al., 2001; Tatsuo and Yanagi, 2002). This particular receptor is expressed on activated T cells, immature thymocytes, memory T cells, a proportion of B cells, activated monocytes, and dendritic cells (Cocks et al., 1995; Sidorenko and Clark, 1993; von Messling et al., 2004). A mutational analysis based on the data available for SLAM-dependent cell entry in Measles virus (Vongpunsawad et al., 2004) demonstrated that residues 526, 527, 528, 529 and 547, 548, 552 located in two nearby clusters in a new CDV H structural model are necessary for efficient canine SLAM-dependent membrane fusion (von Messling et al., 2005). Although some of the unique amino acid substitutions observed in Colombian CDV strains are located near the SLAM-binding region (ie: T544S and E582K), none of them correspond to residues known to be essential for SLAM dependent membrane fusion. However, it is possible that other amino acids support interactions with different receptors (Santiago et al., 2002).

Dog nectin-4 can be used by CDV to gain entry into epithelial cells, and facilitate virus spread (Noyce et al., 2013). In a site-directed mutational analysis, Langedijk et al. (2011) identified 11 residues in the CDV H protein that regulated fusion activity primary canine epithelial keratinocytes (478, 479, 537, 539, 494, 454, 460, 496, 510, 520, and 522). Interestingly, all Colombian wildtype viruses, except strains 39-CO/12 and 14-CO/12, showed a unique substitution from isoleucine to threonine in residue 522 (Figure 13). Whether this non-conservative change, produced by a single nucleotide change (T to C), could alter to some extent the H protein binding to its putative receptor in epithelial cells or modify the tropism of these strains in some way should be addressed using site-directed mutagenesis studies.

N-linked glycosylation is a postraslational modification essential for correct folding, transport, and function of paramyxovirus fusion and attachment glycoproteins (Hu et al., 1994; McGinnes and Morrison, 1995; Segawa et al., 2003). This process involves the addition of N-glycan chains to asparagine (N) residues in the endoplasmic reticulum (ER) at the consensus sequence N-X-S/T, where "X" can be any amino acid except proline (Taylor and Drickamer, 2006). CDVs studied here have 8 potential glycosylation sites (N-X-S/T) at positions 19-21, 149-151, 309-311, 391-393, 422-424, 456-458, 587-589 and 603-605 of the H protein that are common to other wild-type strains from other lineages (Figure 13). Compared to Ondeersteport strain, the H proteins of Colombian CDV viruses have two additional potential glycosylation sites. Biochemical characterization of the H protein has revealed that only two of the up to five additional sites present in wild-type strains are used; however, the N-glycosylation pattern is tightly related to virulence, since recombinant viruses expressing N-glycan-deficient H proteins no longer caused disease, even though their immunosuppressive capacities were retained (Sawatsky and von Messling, 2010). This explains why vaccine strains have been modified to lose potential N-glycosylation sites (Figure 13) through attenuation and are no longer virulent for their hosts. Colombian CDV strains do not present any additional N-glycosylation sites when compared to other wild-type viruses, therefore no assumptions regarding differential virulence related to the N-glycosylation pattern can be made.

5. CONCLUSIONS

Our findings suggest that there is a novel lineage of CDV circulating in Colombian domestic dog populations and that these wild-type viruses are clearly distinct from vaccine strains and from other known lineages; therefore, there is an urgent need for assessing the level of protection against these new CDV variants elicited by vaccine strains through well conducted antigenicity studies. This information portraits an interesting scene for molecular epidemiology of CDV in South America, as it shows that some of the viruses co-circulating could be imported from other countries, (ie. Uruguayan and Brazilian CDV strains are related to European strains), situation attributed to a possible common origin of the viruses or a continuous genetic homogenization due to the commercial exchange between these regions (Panzera et al., 2012). Colombian CDV strains on the other hand, show no ancestral relation with previously characterized strains, either from domestic or wild carnivores.This first genetic characterization of CDV in Colombia underlines the necessity of characterizing CDV strains in other cities of Colombias as well as neighboring countries such as Ecuador, Venezuela and the Guianas, in orden to make inferences at the supranational level.

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