CO-CIRCULACIÓN DE DOS LINAJES DEL VIRUS DEL DISTEMPER CANINO EN COLOMBIA

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Dedicatoria

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CONTENIDO

Lista de abreviaturas

BIC: Criterio de información bayesiana

BLAST: Herramienta de búsqueda de alineamiento local básico.

cDNA: Ácido desoxirribonucleico complementario.

CDV: Virus del distemper canino.

CDV-H: Hemaglutinina del virus del distemper canino.

dN: sustituciones no sinónimas

dS: sustituciones sinónimas

ESS: tamaño efectivo de la población

F: Proteína de fusión.

Fsp: Péptido señal de la proteína de fusión.

FUBAR: Aproximación bayesiana rápida sin restricciones

GliaR: Receptor de las células gliales.

GTR: Tiempo general reversible

H: Hemaglutinina.

HKY: Hasegawa-Kishino-Yano

HPD: Mayor densidad de probabilidad.

IFN- γ: Interferón gamma.

L: Polimerasa viral.

M: Proteína de matriz.

MCC: Máxima credibilidad de los clados

MCMC: Cadena Markoviana Monte-Carlo

ML: Máxima verosimilitud

N: Nucleocápside.

NCBI: Centro nacional para la información biotecnológica.

NJ: Neighborn Joining

P: Fosfoproteína.

PCR: Reacción en cadena de la polimerasa

RNA: siglas en inglés de ácido ribonucleico -Ribonucleic acid-.

RT: Transcriptasa reversa.

RT-PCR: Transcriptasa reversa - reacción en cadena de la polimerasa.

SLAM: Molécula de activación y señalización de linfocitos.

STAT1/2: Señal de traducción y activador de transcripción 1/2.

T92: Tamura de 3 parámetros

tMRCA: Tiempo del ancestro común más reciente.

UTR: Regiones no traducidas.

1.RESUMEN GENERAL

El virus del distemper canino (CDV) afecta los sistemas respiratorio, gastrointestinal, tegumentario y nervioso en perros domésticos y animales silvestres en más de 20 familias, incluso infecta a células humanas en condiciones *in vitro.* La clasificación filogenética con el gen de la Hemaglutinina reporta 17 linajes con un patrón de distribución geográfico.

En el año 2012 en Medellín, se realizó la primera caracterizaron a nivel molecular las cepas del virus del distemper canino (CDV) circulantes en Medellín, las cuales se agruparon en un linaje diferente a los previamente reportados, así este linaje fue denominado Suramérica 3, el cual se considera endémico.

Como continuación de este trabajo, se planteó el objetivo de caracterizar filogenéticamente las cepas de CDV circulantes en Colombia por medio de secuencia extendida, para lo cual se recolectaron muestras de pacientes infectados con CDV en Bucaramanga y Medellín; se obtuvieron secuencias de los genes H , F y genoma completo por el método de Sanger, adicionalmente se contó con la colaboración de la Dra. Paola Barato al suministrar las secuencias del gen F de cepas obtenidas en la ciudad de Bogotá en 2015; una vez obtenidas las secuencias estas se ensamblaron con el programa bioinformático Seqman, los análisis filogenéticos se llevaron a cabo con el programa MEGA 7 usando los métodos de Neighbor Joining, Maximum Likelihood y Bayesiano, además se establecieron sitios bajo selección, recombinación, tasas evolutivas y presencia de mutaciones del CDV Linaje Suramérica 3 asociadas a la adaptación a células humanas, salto de la barrera de especies y patogenicidad por medio de los programas Datamonkey, RDP4, Simplot y BEAST. Los resultados obtenidos evidenciaron la circulación en Medellín de dos linajes diferentes: Suramérica 3 en Medellín, Bucaramanga y Bogotá y un nuevo linaje no descrito en el país el cual se encuentra evolutivamente relacionado con cepas reportadas en Ecuador y en fauna silvestre en los Estados Unidos el cual denominamos Suramérica/Norteamérica- 4. Además, se reporta la recombinación homóloga entre linajes co- circulantes. Con esta información se concluye que el CDV en Colombia posee una de las mayores diversidades genéticas reportadas en el mundo, ya que co-circulan los Linajes Suramérica-3 y Suramérica/Norteamérica-4, además el CDV posee múltiples sitios con selección positiva y recombinación, mecanismos

involucrados en la evolución de este agente infeccioso, ampliando la posibilidad de que pueda convertirse en un problema tanto de salud animal para poblaciones domésticas y silvestres como llegar a ser un potencial problema de salud pública para poblaciones humanas.

2. INTRODUCCIÓN GENERAL

El distemper canino es una enfermedad viral sistémica, altamente contagiosa de distribución mundial que afecta principalmente a cánidos domésticos y salvajes entre otras especies de diferentes Familias como *Procyonidae, Mustelidae, Hyaenidae, Ursidae, Viveridae, Cricetidae, Cercopithecidae, Suidae, Elephantidae* y *Myrmecophagidae* (Martinez-Gutierrez and Ruiz-Saenz 2016; Lunardi et al. 2018)

El virus del distemper canino (CDV) pertenece a la familia *Paramyxoviridae*, género *Morbilivirus*, el cual tiene estrecha relación con el virus del sarampión y la peste bovina. Se caracteriza por afectar los sistemas gastrointestinal, respiratorio, cutáneo y nervioso en todas las especies. La principal vía de infección es a través de las partículas de aerosol expelidas junto a las secreciones nasales además de las excreciones corporales como orina y heces. Un animal infectado puede eliminar el virus hasta por 90 días (Max J G Appel et al. 1982).

El virus posee un genoma RNA de cadena negativa, no segmentado con aproximadamente 15690 nucleótidos. Posee seis genes que codifican dos glicoproteínas (Hemaglutinina y proteína de fusión) , una proteína de envoltura (matriz), dos proteínas transcriptasa asociadas (fosfoproteína y proteína grande) y una proteína que encapsula el RNA viral (proteína nucleocápside) (de Vries, Duprex, and de Swart 2015)

La Hemaglutinina y la proteína de fusión son esenciales para la infección y fusión con los receptores de las células del hospedero. Para el caso de los caninos, los receptores celulares reconocidos son el receptor Nectina -4 y el denominado SLAM (Signaling Lymphocytic Activacion Molecule); siendo el segundo el más importante ya que hace susceptible al hospedero y el receptor Nectina-4 permite la fusión con la membrana celular y la envoltura viral (MacLachlan and Dubovi 2011)

Múltiples estudios filogenéticos se han desarrollado evaluando la Hemaglutinina (H), la cual muestra una gran variabilidad genética (aproximadamente 10% comparada con los otros genes del CDV) que refleja en un patrón geográfico de distribución mundial del virus, dando así una clasificación de las cepas estudiadas en 17 linajes: América 1 (cepas vacunales), América 2-5,Suramérica 2-3, Tipo Ártico , Tipo Rockborn, Asia 1-4 Africa 1-2, European wildlife, Europa/ Suramérica 1 (Radtanakatikanon et al. 2013; Riley and Wilkes 2015; Martinez-Gutierrez and Ruiz-Saenz 2016; Nikolin et al. 2016; Anis, Newell, et al. 2018)

En Uruguay, Argentina y Brasil se ha reportado la circulación del linaje Europa/Suramérica 1 y otro linaje Suramérica 2, que circula sólo en poblaciones domésticas y salvajes de Argentina (Panzera et al. 2015). En Medellín- Colombia, se ha reportado la circulación del linaje Suramérica 3 con características propias que lo diferencian de los otros 16 linajes reportados a nivel mundial (Espinal, Díaz, and Ruiz-Saenz 2014).

Debido a la dificultad técnica de amplificar el gen H (tamaño del gen -1824 pb- y bajo nivel de transcripción), como alternativa para los análisis filogenéticos se ha propuesto usar una porción codificante (405 pb) del gen F (proteína de fusión). Usando esta metodología, se caracterizaron cepas circulantes en Ecuador, las cuales pertenecen a otro linaje nuevo (Panzera et al. 2014). Asimismo, en la ciudad de Bogotá se realizaron estudios filogenéticos con el gen F, en el cual las cepas caracterizadas pertenecen a un linaje nuevo (Sarute et al. 2013; Panzera et al. 2014; Forero Muñoz and Barato 2016).

La caracterización filogenética con el gen H es de mayor importancia debido a que es el gen que determina el tropismo celular, tiene un 10% más de variabilidad que los demás genes y a nivel evolutivo se ha demostrado que con sustituciones en pocas posiciones puntuales le confieren la habilidad al CDV de realizar salto de la barreara de especie; por lo cual, a nivel mundial la mayoría de los estudios filogenéticos se realizan con este gen. Así, se concluye que hasta el momento son 17 Linajes de CDV establecidos a nivel mundial y que las cepas colombianas caracterizadas con el gen H pertenecen al linaje Suramérica 3 (MacLachlan and Dubovi 2011; Mccarthy, Shaw, and Goodman 2007; Espinal, Díaz, and Ruiz-Saenz 2014; Martinez-Gutierrez and Ruiz-Saenz 2016).

La clasificación de linajes realizada a través del estudio de la secuencia de la Hemaglutinina del CDV ha ayudado al conocimiento de la evolución del virus y a entender parcialmente la dinámica de transmisión del CDV entre fauna doméstica y fauna silvestre. Sin embargo, recientes trabajos a partir del uso de secuencias de genoma completo han ampliado dicho panorama permitiendo entender de forma más adecuada no solo la evolución viral, sino también el hallazgo de rasgos patogénicos que podrían explicar potencialmente las posibles fallas vacunales reportadas en algunas regiones del mundo (W. Li et al. 2014).

La caracterización de los linajes circulantes en el país por medio de la secuenciación del genoma completo permitirá establecer la divergencia de las cepas reportadas en Ecuador y Colombia, mutaciones que le confieran capacidad de adaptación a células humanas,

patogenicidad en caninos y salto de la barrera de especie, además determinar el tipo de evolución, lo cual aportará al conocimiento de la filogenética del virus del distemper canino.

3. OBJETIVOS

3.1 OBJETIVO GENERAL

Caracterizar filogenéticamente las cepas de CDV circulantes en Colombia por medio de secuencia extendida.

3.2 OBJETIVOS ESPECÍFICOS

1. Comparar los genotipos de CDV circulantes en Colombia y Ecuador.
2. Describir la secuencia de los genes F, P y N del CDV Linaje Suramérica 3
3. Explorar las relaciones filogenéticas del CDV Linaje Suramérica 3 en secuencia extendida.
4. Explorar la presencia de mutaciones del CDV Linaje Suramérica 3 asociadas a la adaptación a células humanas, salto de la barrera de especies y patogenicidad.
5. Describir la presencia de sitios de recombinación en la secuencia extendida del CDV Linaje Suramérica 3.

4.CAPÍTULO I

REVISIÓN DE LITERATURA

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Evolution of Canine Distemper Virus: Only due to Hemagglutinin?

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Abstract

Canine distemper virus (CDV) is a virus belonging to the genus *Morbillivirus* within the family *Paramyxoviridae* with a worldwide distribution. CDV spreads to the lymphatic, epithelial, and nervous systems of domestic dogs and wildlife, in at least six orders and over 20 families of mammals. Due to the high morbidity and mortality rates and its broad host range, CDV is a possible cause of extinction of endangered species. The present study aims

to explain the great evolutionary capacity of CDV from the *hemagglutinin* (*H*) gene, which has the highest genetic variability of the viral genome. The protein H determines the cellular tropism by binding to signaling lymphocytic activation molecule (SLAM) and nectin-4 receptors of the host. However, the mutation/substitution rates, presence of glycosylation sites, and events of homologous recombination in other viral genes have not been thoroughly studied, making it necessary to conduct studies of CDV evolution from complete genome sequences. This would help to explain its evolutionary potential, thus providing elements to understand CDV pathogenesis in the various susceptible species, vaccine failures, species jump, and its zoonotic potential. This review will focus on the study of CDV evolution by examining the work done with sequences of the *hemagglutinin* gene and the growing body of studies of the complete genome, exposing the limitations arising from studies of a single gene.

Keywords:

Genome, Evolution, Canine Distemper Virus, *Hemagglutinin* gene, Genotype

Introduction

Canine distemper virus (CDV) belongs to the genus *Morbillivirus* within the family *Paramyxoviridae*, which also includes measles virus, seal distemper virus, small ruminant plague virus, and rinderpest virus, the latter of which is already eradicated (MacLachlan and Dubovi 2011). The virus is pleomorphic (spherical and filamentous shapes), with a size between 150–300 nm in diameter and contains a genome comprising single-stranded, non-segmented ribonucleic acid (RNA) of negative coding sense. The genome comprises 15690 nucleotides (15.6 kb), including six genes organized in separate and non-overlapping transcriptional units coding for six structural proteins (Figure 1): nucleocapsid protein (N), phosphoprotein (P), viral polymerase (L), the glycoproteins hemagglutinin (H) and fusion protein (F), and matrix protein (M) (MacLachlan and Dubovi 2011).

The transcriptional CDV units are separated by untranslated regions (UTRs) that are relatively uniform (107-155 nucleotides), except for the UTR between the M and F proteins,

which has approximately 405 nucleotides. This UTR modulates virulence through the translational control of the protein F (Anderson and von Messling 2008).

The *phosphoprotein P* gene (1524 nucleotides) is highly conserved and encodes a viral polymerase cofactor protein (P), and two nonstructural proteins V and C, which are produced by RNA editing and an alternative start of the translation, respectively (MacLachlan and Dubovi 2011). Protein V suppresses the innate immune response by inhibiting the induction of type I and II interferons and the activity of the NF-kappa B complex in the host cell (Schuhmann, Pfaller, and Conzelmann 2011; Chinnakannan, Nanda, and Baron 2013). In measles virus, it has been established that protein C suppresses the IFN-γ signaling pathway by inhibiting dimerization of the phosphorylated STAT1 protein (Yokota, Okabayashi, and Fujii 2011).

The *large protein (L)* gene (6555 nucleotides) is highly conserved and encodes for the viral polymerase. The *N* gene (1572 nucleotides) encodes for the N protein, which encapsulates the viral RNA (MacLachlan and Dubovi 2011). The P, L, and N proteins together make up the transcription/replication complex, known as ribonucleoprotein (MacLachlan and Dubovi 2011).

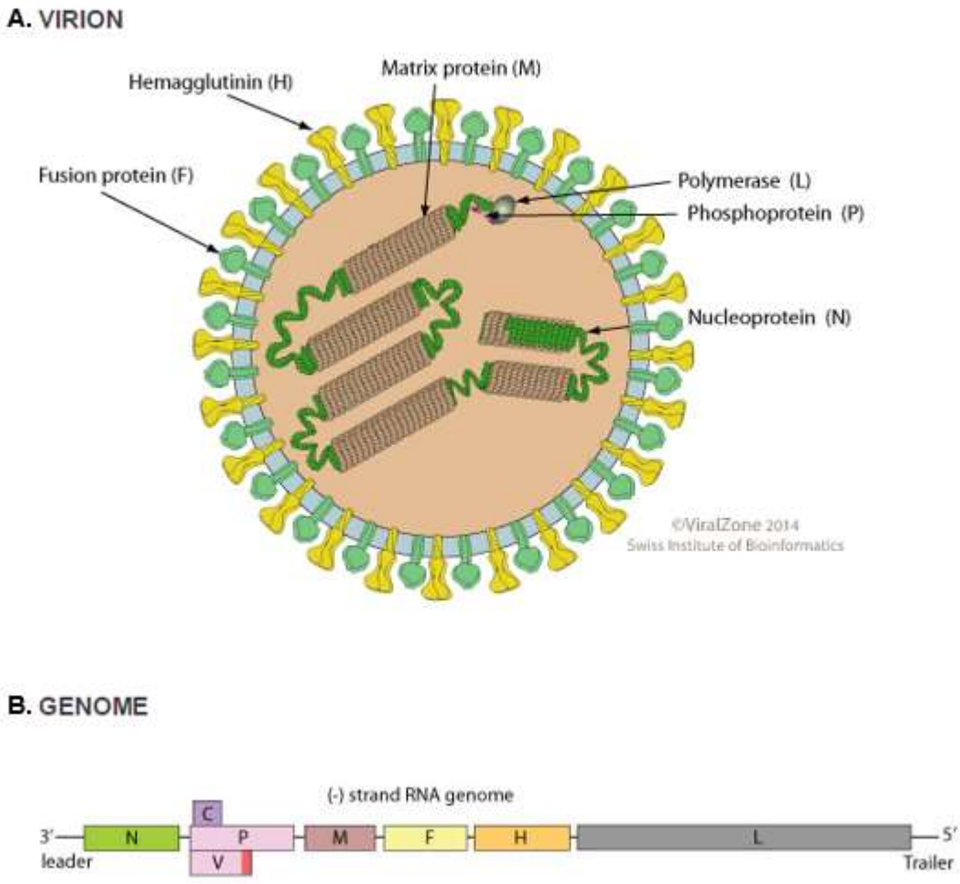

Figure 1. Schematic representation of CDV. (A) Virion highlighting the main proteins. (B) Schematic representation of the CDV genome (with permission. http://viralzone.expasy.org).

The protein M (encoded by the *M* gene with 1008 nucleotides) constitutes the inner layer of the viral envelope and interacts with the cytoplasmic domains of the H and F proteins. In addition, this protein is associated with the ribonucleoprotein complex and is formed in the cytoplasm during the replication cycle, connecting the envelope with the ribonucleoprotein complex, and playing an essential role in viral shedding (MacLachlan and Dubovi 2011).

The fusion protein (F) is encoded by the *F* gene, composed by 1989 nucleotides. After the binding of the protein H to the cellular receptor, F mediates the fusion between the viral envelope and the host plasma membrane at physiological pH (MacLachlan and Dubovi 2011). A short region of this gene (405 nucleotides) encodes for the F signal peptide; the signal peptide region is highly variable and has been used in phylogenetic classification, representing a rapid method for characterizing circulating CDV strains (Sarute et al. 2013).

The *H* gene has 1824 nucleotides and encodes for the hemagglutinin protein, which shows the highest variability among morbilliviruses (Pomeroy et al. 2008). This protein determines the viral tropism and initiates the infection by binding to the receptor SLAM in immune sentinel cells and nectin-4 recpetors in epithelial cells (Tatsuo, Ono, and Yanagi 2001; Noyce, Delpeut, and Richardson 2012). After infection, neutralizing antibodies against the protein H confer immunity for life (M. J. Appel and Summers 1995). Field and vaccine strains are extremely divergent (7-10% for nucleotides, and 8-11% for amino acids); this feature is used for the phylogenetic classification of CDV circulating strains worldwide, which are grouped into 17 lineages with a defined geographical distribution pattern (Bolt et al. 1997; Riley and Wilkes 2015; Martinez-Gutierrez and Ruiz-Saenz 2016).

Disease description: reports of outbreaks in domestic canids and wildlife

Canine distemper is a systemic, highly contagious viral disease, with high morbidity and mortality rates. CDV infection occurs in two phases; first, an acute infection of the lymphatic system; and second, an invasion of epithelial cells, with later viral shedding allowing its transmission by excretion. CDV can also invade the central nervous system (V. von Messling et al. 2003; Rudd, Cattaneo, and von Messling 2006). The disease manifests as depression, anorexia, nasal and ocular mucopurulent secretions, gastroenteritis, and hyperkeratosis of the plantar pads and snout. In addition, seizures, myoclonia with hyperesthesia, ataxia, paresis, and tremors indicate central nervous system involvement. Puppies develop tooth enamel hypoplasia after CDV infection (MacLachlan and Dubovi 2011).

The main infection route is through the aerosolization of nasal secretions and body excretions such as urine and feces (Elia et al. 2015). Patients with subclinical infection can excrete the virus, increasing the chances of disease dispersion in a vulnerable population (Max J. G. Appel et al. 1982).

CDV infects multiple species within the order Carnivora, including domestic dogs (*Canis lupus familiaris,* family *Canidae*), and various wild species within *Canidae* and other families such *as Felidae, Procyonidae, Mustelidae, Hyaenidae, Ursidae,* and *Viverridae.*

Some studies have also reported CDV infection in other mammalian families, such as *Cricetidae, Cercopithecidae, Suidae*, and *Elephantidae* (Martinez-Gutierrez and Ruiz-Saenz 2016). Even, it has been recently reported the participation of CDV in the development of a neurological disease in order Pilosa, family *Myrmecophagidae*, in a southern tamandua (*Tamandua tetradactyla*) from Midwestern Brazil (Lunardi et al. 2018)

Viruses with a broad host range such as CDV are of great importance in the conservation of endangered species because by infecting several species, they can persist in those with large populations and cause disastrous epidemics in endangered species (Seimon, Miquelle, and Chang 2013; Gordon et al. 2015; Feng et al. 2016).

CDV transmission between domestic dogs and wildlife depends on the dynamics between their populations, molecular adaptations of the virus,, host receptors, and other factors that are not fully explained (Viana et al. 2015; Mccarthy, Shaw, and Goodman 2007; Ohishi et al. 2014). Multiple epidemiological reservoirs can lead to the presence of CDV in wildlife even in the absence of infection in domestic populations (Martinez-Gutierrez and Ruiz-Saenz 2016).

Regarding CDV ecoepidemiology, some studies have reported drastic reductions worldwide in the number of individuals from populations affected by CDV in wild areas, and this is a cause of extinction in endangered wildlife species (Terio and Craft 2013). For example, CDV has been reported in near-extinct species, such as in 2014 where it was reported in the giant panda (*Ailuropoda melanoleuca*) in a wild animal rescue and conservation center in China with a morbidity of 27% and mortality of 23% (Feng et al. 2016). CDV has also been reported in monkey breeding sites (*Macaca fuscata* and *Macaca mulata*) with an incidence of 20-60% and a mortality of 5-30% in 10,000 infected animals (Qiu et al. 2011). In Serengeti National Park, Africa, CDV has been reported in four of the five wild canid species, with fatal outcomes of the infection. In 1994 in this park, approximately one third of the African lion population (*Panthera leo*) died in a CDV outbreak (Roelke-Parker et al. 1996); in 2015 in a nature reserve in South Africa, another outbreak occurred in a population of lions with 95% mortality (Loots et al. 2017). In Ethiopia, a mortality of 43-68% caused by CDV was reported

in the years 2005-2006 and 2010 in the Ethiopian wolf *(Canis simensis),* one of the most endangered canid species in the world (Gordon et al. 2015). The molecular epidemiology of CDV in the other 19 non-canid carnivore species present in these ecosystems is unknown (Nikolin et al. 2016).

In the United States, a mortality rate of 45% was reported in free-living raccoons (*Procyon lotor)* present within, around, and outside a zoo in 2001 (Lednicky et al. 2004). CDV has been stated as one of the most important causes of mortality in the gray fox (*Urocyon cinereoargenteus*) (Davidson et al. 1992). Likewise, a CDV outbreak almost led to the extinction of the black-footed ferret (*Mustela nigripes*) (Williams et al. 1988).

Epidemiological studies have shown high rates of CDV seroprevalence in the wildlife in Spain, Portugal, Italy, and Germany, specifically a seroprevalence of 4 to 30.5% in red foxes (*Vulpes vulpes*) from Europe has been reported (Denzin, Herwig, and Van Der Grinten 2013; Lopez-Peña et al. 1994; Martella, Elia, and Buonavoglia 2010; Seimon, Miquelle, and Chang 2013). Additionally, in Brazil, it has been reported a 40.2% prevalence of CDV-positive domestic dogs between 2008 and 2012 because of the high number of stray dogs and low socioeconomic status of canine owners. In most countries of Central and South America, there is little information on CDV epidemiology in domestic dogs (Budaszewski et al. 2014) or CDV exposure in wild cats (Avendaño et al. 2016) or wild canids (Deem et al. 2000).

Although CDV has been linked to the mass death of wild species both free-living (Goller et al. 2010) and in captivity (T C Harder et al. 1996), CDV can also occur in different domestic or wild species without clear clinical symptoms. For example, domestic dogs can be CDV-positive without developing clear symptoms (Greene and Appel 2006; Budaszewski et al. 2014). On the other hand, the experimental inoculation of cats (*Felis silvestris catus*, family *Felidae*) and domestic pigs (*Sus scrofa domesticus*, family *Suidae*) with a CDV strain virulent for domestic dogs resulted in infection and viral replication in lymphoid tissues and macrophages, but without clinical symptoms or virus shedding (M. J. Appel and Summers 1995). Interestingly, the inoculation of two cats with a strain that was lethal in a captive Chinese leopard *(Panthera pardus orientalis,* family *Felidae*) resulted in viremia in

peripheral blood mononuclear cells and mild transient leukopenia and lymphopenia, but without clinical symptoms (T C Harder et al. 1996). In domestic ferrets *(Mustela putorius furo*, family *Mustelidae*), inoculation with an attenuated strain (V. von Messling et al. 2003; B. Sawatsky and von Messling 2010) and with a virulent CDV strain in peccaries (*Pecari tajacu*, family Tayassuidae) resulted in serological conversion without clinical symptoms. In the latter case, only one in three individuals had typical distemper symptoms (M. J. G. Appel et al. 1991).

Virus detection and the recognition of signs and symptoms may be challenging in wild free-living species, which will help to eliminate biases in the selection of species to be included in studies (Dobson and Foufopoulos 2001). However, long-term multidisciplinary research that tracks certain populations and their pathogens has helped to detect several pathogens and the presence or absence of symptoms in wild species (Leendertz et al. 2006; Nikolin et al. 2016). For example, 'silent' CDV epidemics have been observed in the Serengeti-Mara ecosystem, where serological analyses have shown an increase in the exposure to CDV in spotted hyenas and lions without obvious clinical symptoms, high mortality, or a reduction in population size, as observed during the lethal epidemic of 1993/1994 in these species (Harrison et al. 2004; Cleaveland et al. 2007; Munson et al. 2008; Viana et al. 2015). Hyenas from the Maasai Mara National Reserve were more exposed to CDV than those near human settlements, showing that in this case, domestic dogs may not be the only animals that transmit CDV to this wild species (Harrison et al. 2004).

These results show the importance of long-term constant CDV monitoring in target populations, regardless of whether symptoms are observed. In this way, the complex scenarios of CDV transmission may begin to be understood, where certain strains may be virulent in some species but not in others (Nikolin et al. 2016). Therefore, the epidemiological importance of asymptomatic individuals in the spread of the disease should be understood (Budaszewski et al. 2014), as should the existence of dead-end hosts—which are asymptomatic and do not shed the virus—such as the cat and pig (M. J. G. Appel et al. 1974); this is also proposed for the hyena and lion when infected with CDV canid strains (Nikolin et al. 2016). Integrated research incorporating the experience of wildlife managers,

behavioral and conservation biologists, veterinarians, virologists, and immunologists (among other scientific areas) and including several wild and domestic species is essential for understanding the complex epidemiological dynamics of CDV in multiple hosts.

The lethal epidemics of CDV in multiple wild species, including vulnerable species (Goller et al. 2010), have increased the understanding of the capacity of this virus to cause high mortality in carnivores, causing a negative impact on wild species, particularly those at risk of extinction (Woodroffe et al. 2004). It is necessary to implement strategies to mitigate the infection, such as limiting contact between wild and domestic species (van Heerden et al. 1995) or administering antiviral vaccines to reduce the strong impact of CDV on the populations at risk of extinction (Martinez-Gutierrez and Ruiz-Saenz 2016). Although there are successful examples of vaccination against CDV in several carnivore species (Williams et al. 1988; Borges et al. 2001; Coked et al. 2005; Feng et al. 2016), there are also multiple cases where, after vaccination, typical CDV symptoms have appeared with fatal consequences in different wild carnivore species (Halbrooks et al. 1981; Kazacos et al. 1981; Thomas-Baker 1985; Davidson et al. 1992), including species at risk of extinction (Bush et al. 1976; Carpenter et al. 1976; McCormick 1983b; Van Heerden et al. 1989; Durchfeld et al. 1990; van de Bildt et al. 2002; Fen et al. 2016).

Although these diverse examples do not allow differentiating between vaccine failure—a vaccine that does not provide protection against wild CDV strains—or possible vaccine escape these cases question the efficacy of vaccination in multiple wild carnivore species. As the effectiveness of different vaccines (adapted modified live vaccine in canid cells or in eggs) can vary even between closely related species (Halbrooks et al. 1981; Davidson et al. 1992), the efficacy, safety, and possible risks of administering vaccines to free-living wildlife should be critically evaluated to avoid possible lethal consequences, especially in areas of high biodiversity. Moreover, negative immunological consequences may be caused by the high stress created when handling animals for vaccination (Burrows, Hofer, and East 1994; Burrows, Hofer, and East 1995) and by the vaccine (McCormick 1983a), which may leave the animals susceptible to CDV and other pathogens.

Host diversity of CDV: domestic canids and wild species

By analyzing the *H* gene and determining the most recent common ancestor, it has been proposed that the origin of CDV can be traced back to the United States in 1886; from there, it has spread throughout the world. Panzera et al. reported in 2015 that this ancestor was divided into two major clades: the first in 1923 originated the lineages Europe-3, South Africa, Asia-2, South America-2, North America-2, Europe/South America-1 and Asia-1; the second in 1978 was limited to Asia (Panzera et al. 2015).

The ability of CDV to jump between species is clear when observing the disease in different orders and families of mammals worldwide. By analyzing lineages (internal nodes), Panzera et al. found that the probability of CDV first infecting domestic canids and then wildlife is higher than in the opposite case, with a value of 0.73 of probability ; thus, domestic canids are probably the main virus reservoir worldwide (Panzera et al. 2015).

Although the initial spread of CDV resulting from the interactions between domestic canids and wildlife has been shown to trigger high mortality in wildlife (Viana et al. 2015), CDV spreading from wildlife to domestic canids is also possible (Beineke, Baumgärtner, and Wohlsein 2015). This raises the possibility of another reservoir in wild populations because the analysis of the CDV outbreak in lions in Serengeti National Park of 1994 shows the outbreaks in domestic canids were asynchronous with those in lions, making it possible to suspect the infection may persist in other wild species interacting in this ecosystem (Viana et al. 2015; Martinez-Gutierrez and Ruiz-Saenz 2016).

Due to the lack of information of the ecoepidemiology of the virus in these wild ecosystems, it is necessary to look for reservoirs of CDV in peridomestic and mesocarnivores wildlife because seropositivity has been reported in clinically healthy animals belonging to various orders and families, showing that virus circulation in wild ecosystems occurs through certain species acting as a "meta-reservoir"; these wild animals transmit the virus to diverse interconnected populations, explaining the asynchronous outbreaks reported in these ecosystems (Martinez-Gutierrez and Ruiz-Saenz 2016). Proof of this, is the report of CDV

in hyenas and cheetahs months after a CDV outbreak in lions of South Africa in 2015 (Loots et al. 2017). This same scenario occurs in the United States; however, the raccoon has already been identified as the second CDV reservoir. The raccoon presents peridomestic habits, having contact with infected dogs, thus spreading the infection to other wildlife in wild ecosystems and to healthy dogs in urban ecosystems (Riley and Wilkes 2015).

The study of the factors determining viral host range is critical for the understanding of the diversity, evolution, and emergence of this virus and for the potential to predict virus changes in the host (Taylor, Latham, and Woolhouse 2001; Woolhouse, Taylor, and Haydon 2001; Pulliam 2008; Pepin et al. 2010). Mutation is a molecular mechanism generating genetic variation—on which random drift and natural selection act—giving shape to the genetic structure of virus populations, and thus being one of the dominant forces driving virus evolution (Domingo and Holland 1997).

Virus adaptability to different environments can be visualized as an adaptive landscape in which the peaks and valleys represent fitness estimates of the relative ability to enter and replicate successfully in an environment, such as in a different host species (Domingo and Holland 1997). Functional mutations allow the virus to move by transmission fitness, and selection contributes to correct adaptive mutations (Lauring, Frydman, and Andino 2013; Holmes 2009). CDV cross-species transmission can be viewed in an evolutionary model in which there is a fitness gradient between the donor and recipient species (Figure 2. The two peaks represent the optimized viral fitness of specific donor mutants and the new recipient species and the specific mutations required to reach the optimum in the new host (Parrish et al. 2008; Kuiken et al. 2006). Here, viral genetic variation is studied in host coevolution to understand the degree to which hosts modify virus diversity. Mutations optimizing the CDV ability to successfully infect a new host can reduce its fitness in an alternative host, a phenomenon known as antagonistic pleiotropy (Elena, Agudelo-romero, and Lali 2009).

Gradient differences between peaks and valleys show the level of fitness compensation between the two hosts. A comparative genetic study with several complete CDV genomes has shown that some residues are under positive selection, all in the hemagglutinin protein

(CDV H), which is the viral protein interacting directly with the SLAM receptor in immune cells (Mccarthy, Shaw, and Goodman 2007). Within the CDV H receptor binding region, substitutions for one of these residues (site 549) were systematically observed in non-canid hosts, signifying their importance in host adaptation (Figure 2b, McCarthy et al., 2007; Nikolin et al., 2012a). The hypothesis is that even when few substitutions are needed for cross-infection, the probability of extinction of "partially adapted" viruses should be high because their fitness is not optimal in any of the hosts, and therefore, these strains would be overcome by strains with greater fitness (Parrish et al. 2008). However, for CDV, fitness analysis for evaluating the impact of substitutions at site 549 for viral entry and replication in canid and non-canid hosts revealed an alternative scenario for the reconstruction of viral evolution when considering fitness compensations between hosts: the evolution of specialist and generalist traits (Nikolin, Osterrieder, et al. 2012).

In a homogeneous environment with a single host species, strong coevolution promotes the optimization of viral fitness in that host (i.e., fitness peaks), resulting in low fitness if the virus transfers to other species (i.e., fitness valleys). Therefore, specialists should be favored in homogeneous environments (Elena, Agudelo-romero, and Lali 2009). In heterogeneous environments with several hosts, the evolution of generalists with the highest average fitness allowing the virus to infect all the hosts should be favored, which finally results in suboptimal fitness in any individual host in comparison with specialists in a single species (Kassen 2002). Therefore, specialists should have greater fitness in the host species and be more adapted than generalists (Elena and Lenski 2003).

In CDV, the large (and growing) world population of domestic dogs might represent such a favorable and homogeneous environment that would allow optimizing CDV fitness in this host. Fitness tests showed that dog CDV H showed higher performance in cell lines expressing canid SLAM than non-canid (African lion and domestic cat), which points to fitness compensation. On the other hand, non-canid CDV H behaved equally well independently of host SLAM (canid or non-canid), but the overall performance was lower than that of CDV H of the domestic dog in the cells expressing domestic dog SLAM. In

addition, the substitution of site 549 from a canid to a non-canid residue in dog CDV H showed a fitness reduction in domestic dog cells (Nikolin, Osterrieder, et al. 2012).

The results of comparative genetic analysis and CDV fitness tests on canid and non-canid species allow another view of cross-species virus transmission (Figure 2) that is consistent with the presence of specialist features (for example, fitness peaks in domestic dogs) and those of generalists (shallow fitness valleys, possibility to infect several species) (Figure 2b) in the relationship between CDV H and the SLAM receptor of canid and non-canid hosts.

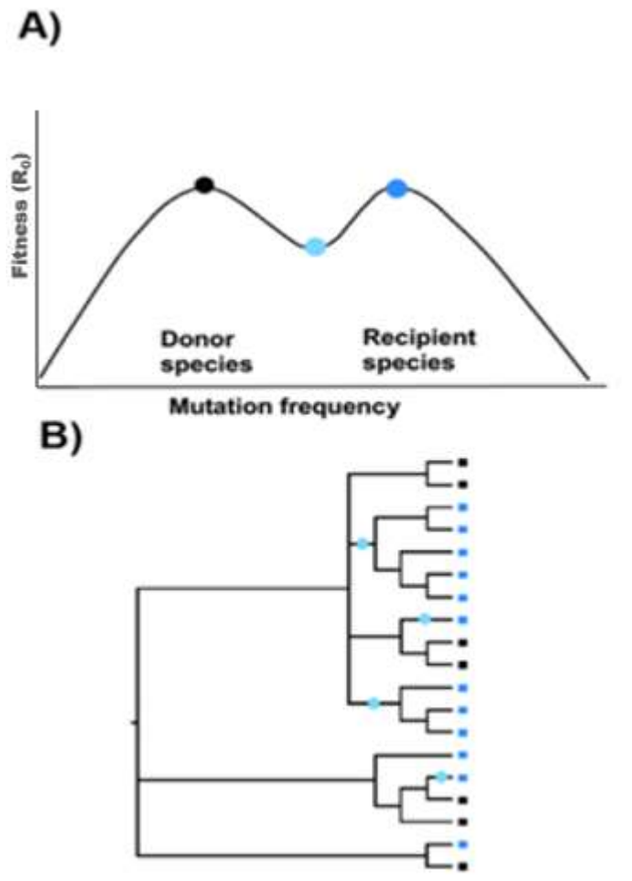

Figure 2. Evolutionary model for cross-species transmission of CDV. In the model, each peak represents the optimized viral fitness in a given host species, and specific mutations (colored circles) are essential to reach this optimum in the new host. (A) The two fitness peaks of CDV are separated by a shallow fitness valley, thus few adaptive mutations are required. One mutation allows the infection of other species but also decreases the fitness in the donor species (light blue circle, generalist trait). Coevolution with the new species can select for further mutations that optimize the fitness (dark blue circle, specialist trait). (B) CDV infection in canids and non-canids as an example of the model presented in (A). A simplified phylogeny of the *H* gene showing the relationships between CDV strains from

canid and non-canid hosts, indicated at the tip of the tree as black and blue squares, respectively. The distribution of the amino acid site 549, which has been shown to be involved in CDV infection in non-canid hosts and is under positive selection, is mapped onto the branches of the tree. Commensurate with the coloring in panel (A), the substitution of site 549 is shown in light blue because it is considered a generalist trait that permits the infection of various non-canid species, but with lower fitness than the specialist trait in canids, marked in black. Panel A was adapted from Parrish et al. 2008. Panel (B) was adapted from McCarthy et al. 2007.

CDV and measles virus

CDV and measles virus belong to the genus *Morbillivirus*, presenting the same genomic organization. The high homology along nucleotidic and amino acidic sequences result in high levels of functional and structural conservation in morbilliviruses, which have been often used in genetic engineering to develop vaccines and therapeutics (Budaszewski da Fontoura and von Messling 2016).

Previous studies have shown that morbilliviruses circulate in nature (Lin and Richardson 2016) by using two primary receptors: SLAM, expressed in cells of the immune system and in activated T and B cells (Ohishi et al. 2014), and nectin-4, expressed in polarized epithelial cells (Mühlebach et al. 2011; Noyce, Delpeut, and Richardson 2012) (Noyce, Delpeut, and Richardson 2012). The SLAM receptor is the main morbillivirus receptor because it allows its initial entry into cells of the immune system (Budaszewski da Fontoura and von Messling 2016), where four interaction sites for measles and CDV in the amino acidic sequence of SLAM have been identified: 123, 127, 129, and 131 (Bieringer et al. 2013; Ohishi et al. 2014; Lin and Richardson 2016).

The nectin-4 receptor is involved in morbilliviruses cell-to-cell propagation and cell-cell fusion (syncytia formation) (Noyce, Delpeut, and Richardson 2012; Delpeut, Noyce, and Richardson 2014). Four binding sites on the nectin-4 receptor have been identified for CDV H: F132, P133, A134, and G135 (Delpeut, Noyce, and Richardson 2014). This receptor has

been associated with neurovirulence of CDV in dogs, and besides to being present in epithelial cells, is also present in neurons (Pratakpiriya et al. 2017). In humans, the nectin-4 receptor is not present in neurons, but is present in epithelial cells (Lapp et al. 2014). Despite these findings, other authors could not confirm the presence of nectin-4 in the white matter or astrocyte cultures of dogs, but when challenging an astrocyte culture with CDV with modified binding to the nectin-4 receptor, they obtained growth and spread of CDV in canid astrocytes, in which there was neither cytolysis nor cell fusion, but observed neighboring infected astrocytes. This suggested a third unknown cellular receptor and cell-to-cell viral transmission (non-cytolytic infection). The receptor was designated glia R, which other authors have reported as the means of colonization of other morbilliviruses in the brain of dolphins. However, all authors conclude that this new receptor should be characterized because it is very important for the neurovirulence of CDV and other morbilliviruses (Alves et al. 2015; Di Guardo, Giacominelli-Stuffler, and Mazzariol 2016).

Due to CDV reports in non-human primates, the ability of the virus to infect humans is subject to speculation; some studies have shown that CDV can gain the ability to infect human cells. With a change of a single amino acid in the H protein after three culture passages, CDV adapted to the human nectin-4 and SLAM receptors (Bieringer et al. 2013). Another study conducted by Otsuki et al.2013 showed that after an amino acid substitution in each of the V/C, F, and H proteins and 8 culture passages, CDV adapted to both of the human receptors (Otsuki, Nakatsu, et al. 2013).

At the receptor level, humans are susceptible to CDV infection (Otsuki, Nakatsu, et al. 2013; Bieringer et al. 2013). Studies of non-human primate models show a high neuropathogenic potential by reporting severe encephalitis in these animals (Fen et al. 2016) and possible associations with multiple sclerosis by finding CDV antibodies in patients with this disease, strengthening the hypothesis of co-infection with CDV, and therefore, the chronicity of the neurological panorama, as these two diseases have been associated since the mid-1990s (Bertus K. Rima and Duprex 2006; Rohowsky-Kochan, Dowling, and Cook 1995).

Regarding CDV zoonotic potential, humans present cross-reactive antibodies against CDV due to vaccination against measles, which might protect against CDV infection, or at least do so in non-human primate models (Lin and Richardson 2016; Zhang et al. 2015). However, if measles vaccination ceases after a possible measles eradication, CDV could jump to humans and cause disease (Otsuki, Nakatsu, et al. 2013; Bieringer et al. 2013).

Genetic variability of CDV

As mentioned, the protein H determines the cellular tropism when interacting with the SLAM receptor in host lymphoid tissues and with nectin-4 in epithelial tissues; therefore, the *H* gene has been subjected to a greater evolutionary pressure than the other genes. Phylogenetic analyses use this fact to study CDV evolution (Sattler et al. 2014; Fischer et al. 2016).

Studies of the H gene show a worldwide distribution of the genetic drift of CDV, classifying the strains studied into 17 lineages: America-1 (vaccine strains), America-2, America-3, America-4, America-5, Arctic-like, Rockborn-like, Asia-1, Asia-2, Asia-3, Asia-4, Africa-1, Africa-2, European Wildlife, Europe/South America-1, South America-2 and South America-3 (Martinez-Gutierrez and Ruiz-Saenz 2016; Ke et al. 2015) (Figure3).

These lineages are defined according to the amino acid divergence of the protein H between circulating strains. Thus, it is considered that two given strains belong to the same lineage when their amino acid divergence is lesser than 3.5%, and they belong to a different lineage when it is higher than 4% (Martella et al. 2006; Espinal, Díaz, and Ruiz-Saenz 2014). On the other hand, following the guidelines based on measles studies, sub-lineage strains present a minimum "bootstrap" 70% value and at least 98% aminoacidic identity (Budaszewski et al. 2014).

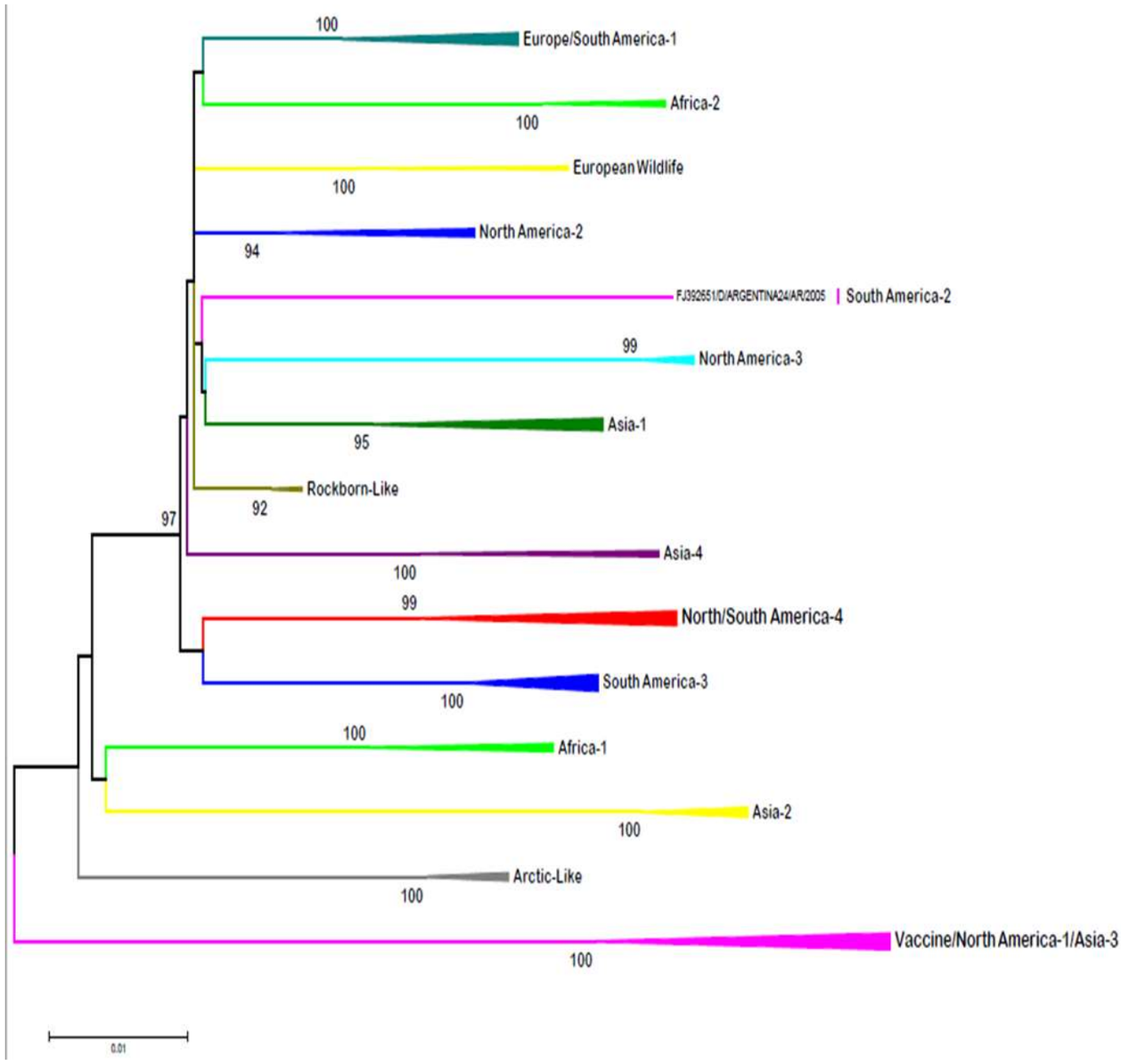

Figure 3. Phylogenetic relationship between 69 CDV strains based on the H gene sequence. The phylogenetic tree was obtained by the Maximum likelihood method with a Bootstrap of 1000 based on the evolution model T92 + G, 69 sequences were analyzed with a total of 1824 nucleotides. Evolutionary analyses were conducted in MEGA7

In 2007, McCarthy et al. established a precedent in the study of CDV evolution by suggesting that the ability of the virus to jump species is due to only two specific site substitutions in the

protein H: E/G530D/N/R and Y549H, which provide better CDV adaptation to the SLAM receptor of different hosts (McCarthy, Shaw, and Goodman 2007).

Later studies confirmed that the positions 530 and 549 present several amino acid substitutions. For example, strains from domestic dogs have eight possible substitutions at position 530 (G/D/N/E/R/S/A/K) and are classified into ten genetic lineages; non-canid wild animals have seven substitutions at position 530 (G/D/N/E/R/C/V) and are classified into seven lineages, while wild canids have three substitutions at position 530 (G/N/R) and are classified into five lineages. For all the species above described, CDV strains present either the residue Y or H at the position 549 (Table 1) (Liao et al. 2015).

Table 1. Amino acids reported at positions 530 and 549 of the CDV *H* protein.

Table 1. Summary of amino acids present at positions 530 and 549 of the *H* protein interacting with the SLAM receptor				
***H* PROTEIN**				
530	**549**	ANIMAL	LINEAGE	AUTHOR
G	Y	Dogs	Europe, Asia 1, America 2, Arctic-like, Asia 2	(Nikolin et al. 2012) (Liao et al. 2015)
G	Y	Wild canids	Europe, Asia 1,	(Nikolin et al. 2012) (Liao et al. 2015)
G	Y	Wild species (other than canids)	Europe, Asia 1,	(Nikolin et al. 2012) (Rentería-Solís et al. 2014) (Liao et al. 2015)
G	H	Wild canids	Europe, Asia 1,	(Nikolin et al. 2012) (Panzera et al. 2015) (Liao et al. 2015)
G	H	Wild species (other than canids)	Europe Asia 1, America 2	(Nikolin et al. 2012) (Panzera et al. 2015) (Liao et al. 2015)
G	H	Dogs	America 2, Europe, South America 1	(Nikolin et al. 2012) (Liao et al. 2015) (Fischer et al. 2016)
D	Y	Dogs	European Wildlife, South America 2	(Nikolin et al. 2012) (Liao et al. 2015)
D	Y	Wild species (other than canids)	European Wildlife, Africa 2	(Nikolin et al. 2016)

D	Y	Rockborn-like, Candur, China, Sweden	Vaccine	(Nikolin et al. 2012) (Liao et al. 2015)
N	H	Wild canids	European Wildlife	(Nikolin et al. 2012)
N	H	Dogs	European Wildlife, America 1	(Liao et al. 2015)
N	H	Wild species (other than canids)	America 1	(Rentería-Solís et al. 2014) (Liao et al. 2015)
D	H	Wild species (other than canids)	European Wildlife, Africa 2	(Nikolin et al. 2012) (Rentería-Solís et al. 2014) (Liao et al. 2015)
C	H	Wild species (other than canids)	European Wildlife	(Nikolin et al. 2012)
N	Y	Dogs	Arctic-like, Africa 1, America 1	(Nikolin et al. 2012) (Liao et al. 2015)
N	Y	Wild species (other than canids)	America 1	(Nikolin et al. 2012) (Liao et al. 2015)
N	Y	Wild canids	Arctic-like	(Liao et al. 2015)
E	Y	Dogs	Asia 2	(Nikolin et al. 2012) (Liao et al. 2015)
E	H	Wild species (other than canids)	Asia 2	(Rentería-Solís et al. 2014)
R	Y	Wild canids	Asia 2	(Nikolin et al. 2012)
R	Y	Dogs	Asia 2	(Liao et al. 2015)
R	H	Wild species (other than canids)	America 2	(Nikolin et al. 2012) (Liao et al. 2015)
R	H	Wild species (other than canids)	America 2	(Rentería-Solís et al. 2014)
S	H		Vaccines	(Nikolin et al. 2012) (Liao et al. 2015)
S	L		Vaccines	(Liao et al. 2015)
S	Y	Dogs	Arctic-like	(Liao et al. 2015)
A	Y	Dogs	Asia 1	(Liao et al. 2015)
V	H	Wild species (other than canids)	European Wildlife	(Nikolin et al. 2012) (Liao et al. 2015)
K	Y	Dogs	Asia 2	(Liao et al. 2015)
Other positions reported in the *H* protein				
***H* Protein**		ANIMAL	LINEAGE	AUTHOR
542	549			
F	Y	Wild species (other than canids)	Asia 1	(Zhao et al. 2014)

Even though the residue 549 reveals the same two substitutions in all the affected species, dogs present a greater tendency for 549Y because they are more likely to be infected with

dog strains, and the 549H substitution occurs mainly in wildlife. This suggests a possible association between this substitution and the species affected (Liao et al. 2015; Nikolin, Wibbelt, et al. 2012).

Likewise, studies conducted in China characterizing different CDV isolates in mink breeding sites (wild species other than canids), raccoon dogs and foxes (wild canids) showed the trend 530G and 549H. In monkeys (wild species other than canids), there was a trend of 530G, 542F, and 549Y, with an additional substitution at another site of the protein H (542F) that might play a role in the inter-species jump (Table1) (Zhao et al. 2014).

However, in an outbreak in raccoons (wild species other than canids) in Europe, researchers found the trend 530G and 549Y, and concluded that the Y549H substitution apparently is not essential to facilitate infection in wildlife other than canids. The explanation for this outbreak was that raccoons are in contact with foxes (wild canids), which act as a wild reservoir and have greater contact with domestic canids, in which the substitution 549Y circulates (Rentería-Solís et al. 2014).

Contributing to this debate, in dogs from Brazil, there was an 11.5% presence of the Y549H substitution. In addition, all the dogs presented 530G and R580, the latter representing 89% of the circulating strains (Table 2). The authors suggest that this trend may be associated with the condition of the "stray" dogs that were sampled who are more likely to have contact with wildlife, which may be wild reservoirs in these ecosystems (Fischer et al. 2016).

Table 2. Amino acid substitutions in the CDV *H* and *V* proteins.

TABLE 2. AMINO ACID SUBSTITUTIONS REPORTED IN THE *H* AND *V* PROTEINS					
Other positions reported in the *H* and *V* proteins					
***H* PROTEIN**		***V* PROT**	**ANIMAL**	**LINEAGE**	**AUTHOR**
519	**549**	**134**			
I	H	S	Wild species (other than canids)	Africa 2	(Nikolin et al. 2016)
R	Y	G	Wild canids	Africa 2	(Nikolin et al. 2016)
R	H	G	Wild canids	Africa 2	(Nikolin et al. 2016)
R	Y	G	Dogs	Africa 2	(Nikolin et al. 2016)
I	H		Wild species (other than canids)	America 2	(Nikolin et al. 2016)
***H* Protein**			**ANIMAL**	**LINEAGE**	**AUTHOR**
530	**549**	**580**			
G	H	Q	Dogs	South America 1	(Fischer et al. 2016)
276	**392**	**542**			

V	R	F	Wild species (other than canids)	Asia 1	(Fen et al. 2016)
530	**519**	**549**			
N	R	Y	Dogs	South America 3	(Espinal et al. 2014)

Position 530 shows ten substitutions in all affected species, differing between canids (domestic and wild) and wild species other than canids: dogs 530S/A/K, and wild species other than canids 530C/V (see Table 1) (Liao et al. 2015).

Recent analyses of the CDV strains responsible for the outbreak in lions of the Serengeti National Park in the 1990s demonstrated that all of the species affected (including dogs and wild canids) presented the 530D substitution (Nikolin et al. 2016), agreeing with Liao et al. who reported the 530 site is not directly related to species jump because it is conserved in different species (Liao et al. 2015).

Regarding to the wildlife, more sequences of the *H* gene are needed to determine the trend of the presence of substitutions at positions 530 and 549, because the 549H substitution has not been reported in the lineages of Asia, South America, and Africa due to the lack of studies in wild species from these regions (Nikolin, Wibbelt, et al. 2012).

Table 3. Amino acid substitutions in the CDV *H* protein reported in strains adapted to human cell lines.

CDV GENES				**AUTHOR**
***V* GENE**	***F* GENE**	***P* GENE**	***H* GENE**	
Y267C	C116Y	M267V	M548T	(Otsuki et al. 2013)
			D540G	(Bieringer et al. 2013)

Regarding species jump to humans, the literature reports that substitutions D540G and M548T in the protein H, besides Y267C in the protein V, C116Y in the protein F, and M267V in the protein P allow cell invasion (Table 3). However, different authors suggest there must be mutations in other genes allowing the intracellular adaptation of CDV (Otsuki, Nakatsu, et al. 2013; Bieringer et al. 2013). We aims to point out that CDV complete genome must be evaluated to help establish other substitutions that can define its capacity to jump between hosts, because earlier studies aimed to explain an adaptation process from a single component

(protein H) ignoring the genetic changes in other regions that may contribute to the inter-species jump.

Concepts such as fitness -defined as ‘the capacity of a virus to produce infectious progeny in a given environment’’- (Domingo and Holland 1997), quasispecies (collections of closely related viral genomes subjected to a continuous process of genetic variation, competition among the variants generated, and selection of the most fit distributions in a given environment) (Domingo, Sheldon, and Perales 2012) , coevolution – defined as “an evolutionary change in a trait of the individuals in one population in response to a trait of the individuals of a second population, followed by an evolutionary response by the second population to the change in the first”- (Janzen 1980) , and antagonistic pleiotropy (in which a beneficial mutation in one environment is either harmful or neutral in another environment, or mutations that are neutral in the environment in which they arose are deleterious in another) (Nikolin, Osterrieder, et al. 2012) represent an explanation of the adaptation and evolution of CDV in its different hosts.

For example, canine distemper is thought to have specialized in two major host orders: Caniformia and Feliformia, in which the virus quasispecies with better host fitness are considered specialized strains because by having the ability to interact with SLAM receptors they have coevolved, fixing mutations allowing it to specialize in a particular host; thus, antagonistic pleiotropy in distemper shows that substitutions at the 549 site of hemagglutinin are related to species jump. However, the capacity for evasion of the immune response should be studied in the other viral genes in each affected host (Nikolin, Wibbelt, et al. 2012; Nikolin, Osterrieder, et al. 2012).

Origin and geographical spread of CDV

As mentioned previously, the geographical origin of CDV is thought to be the United States in 1886. From there, it has been assumed that the virus spread throughout the world in several migratory events resulting in the multiple current lineages. It should be noted that ancient

strains may exist, may be extinct, may not have been isolated from wild hosts, or may not have had their sequences reported in databases; therefore, 1886 represents the best estimation that can be made as to the date of viral origin.

By studying the 208 sequences of the CDV *H* gene obtained from GenBank, it was established that the first migration of the virus occurred in 1923 from the United States to Greenland and migrating from there to Italy in 1960, resulting in the lineages Europe-3/Arctic-like and Europe-3. An older spread in Italy (1949) occurred directly from the American ancestor, subsequently spreading to other European countries, Asia, and the United States, breeding the Europe/South America-1 lineage. From Italy, it arrived in South America through Brazil, and then in Uruguay and Argentina in 1975; these strains belong to the Europe/South America-1 lineage (Figure 4) (Panzera et al. 2015).

In 1950, the American ancestor arrived directly in Argentina, resulting in the South America-2 lineage. In 1955, a new invasion of the American ancestor arrived in Hungary and Austria; from there it spread to Italy and Germany, producing the Europe-2/Wildlife lineage (Panzera et al. 2015).

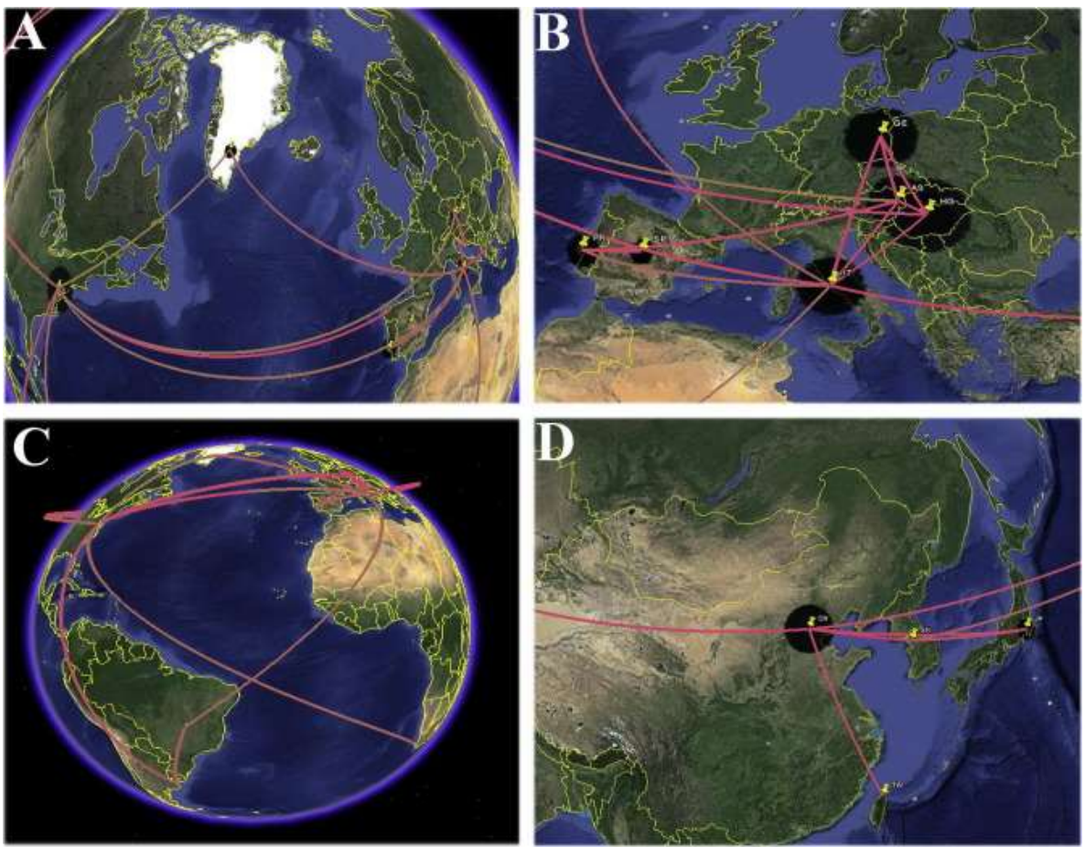

Figure 4. Spatial dynamics of CDV evolution and migration. The images show the most significant migration routes (red lines) of the spread of CDV (A to D). Adapted from Panzera et al. 2015. DOI: 10.1016/j.ympev.2015.06.015.

Another migration occurred in 1926 from the United States and arrived in South Africa, possibly due to animal trafficking to this region, resulting in the African lineage. Another migration occurred in 1928 to Asia (South Korea and Japan), creating the Asia-2 lineage. In 1939, the American ancestor arrived back in Asia through China, possibly during the Second World War, creating the Asia-1 lineage. Once again, the American ancestor migrated, arriving in China in 1978, producing the North America-1 lineage (Panzera et al. 2015).

Evolutionary rates and molecular clock of CDV

Different studies have tried to evaluate the time of the most recent common ancestor (tMRCA) for CDV. The difference in the number of strains available in GenBank has meant that the results can vary between different studies. A first approach, published in 2008, estimated the tMRCA for CDV to be 58 years earlier from a 2001 sequence, suggesting that the ancestor existed in 1943, with an interval from 1894 to 1974 and 95% higher probability density (HPD) (Pomeroy et al. 2008). Next, a tMRCA was determined by analyzing a strain from 2011, which was estimated at 125 years, corresponding to the year 1886, with an interval from 1858 to 1913 and 95% HPD (Panzera et al. 2015). In Taiwan, the tMRCA was 1945, with an interval from 1918 to 1966 and 95% HPD (Ke et al. 2015). Likewise, in Brazil, another tMRCA was found to be 92 years starting in 1919, with an interval from 1899 to 1944 and 95% HPD (Fischer et al. 2016).

The differences found in the tMRCA studies for CDV are attributed both to the difference in the number of sequences evaluated in the different studies (from 35 to 208 sequences of the *H* gene) and to the geographical and temporal diversity of the sequences. In this sense, Panzera's study provides more reliable results by including sequences with greater temporal and spatial divergence in the analysis. However, in this analysis the sequences of the South America-3 lineage are not used. The 2016 study by Fischer et al. showed how the sequences

of the South America-3 lineage circulating in the city of Medellín may have a common origin with the South America-2 lineage, having a common ancestor in the European Wildlife lineage and a divergence estimated in the early 60s (Figure 5) (Fischer et al. 2016).

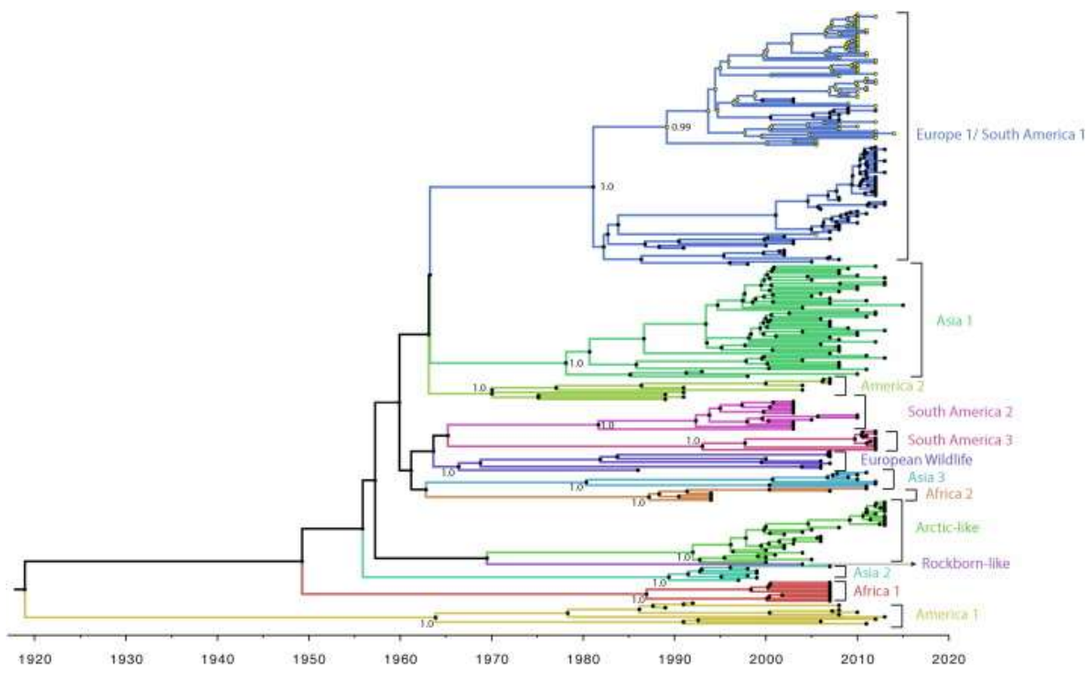

Figure 5. Time-measured Bayesian maximum clade credibility (MCC) tree for the CDV H gene. The Brazilian taxon is marked with yellow circles in the external nodes (representing the available sequences) and in the internal nodes (representing the locations). Tree branches show the a posteriori probability for CDV. The branches are colored according to the clade. Source: (Fischer et al. 2016) DOI: 10.1016/j.meegid.2016.03.029

CDV evolutionary rate, defined as the substitution rate per year reported for the *H* gene is 4.8E-4, with 95% HPD and a range of 3.9E-4 to 5.9E-4, which represents a high evolutionary capacity (Panzera et al. 2015). Other substitution rates have been reported for the *H* gene, including 11.65E-4 and 7.41E-4 substitutions per site annually (Ke et al. 2015; Pomeroy et al. 2008). This shows that substitution rates of CDV are as high as seen for other morbilliviruses such measles (6.5E-4) (Pomeroy et al. 2008). This is related to the high genetic variability and substitution per site that help CDV to adapt to other hosts, which would explain its great ability to cross the species barrier (Nikolin, Osterrieder, et al. 2012).

Pathogenicity predictions

Genetic approaches are one of the multiple tools used to analyze virus spread and pathogenesis. Association predictions between morbidity and lethality, and the presence of substitutions at certain amino acidic positions have been stated by several authors (Ke et al. 2015; Fischer et al. 2016; Nikolin et al. 2016) indicating that those predictions are hypotheses that have not been statistically proven or fully demonstrated biologically.

In the Serengeti wildlife outbreak of 1994 a new amino acidic change was found in the protein H (R519I), which was associated with the presence of clinical symptoms in wild species other than canids. In this study, the authors evaluated various combinations of substitutions at positions 519 and 549 of the protein H in dogs, wild canids, and wild species other than canids. They also described the relationship of these combinations with the presence or absence of morbidity and mortality in the species affected—the combination of 519I and 549H in non-canids wild species caused death, while these combinations were not present in wild canids. The combinations of 519R/549Y and 519R/549H caused death in dogs and wild canids, respectively, but there was no symptomatology in species other than wild canids (Table 2) (Nikolin et al. 2016).

Similarly, every dog with the 549H residue from Brazil died, with a statistically significant association ($p < 0.05$) reported in the study. In addition, the authors concluded that this substitution is associated with the criollo breeds ($p < 0.05$) (Fischer et al. 2016). In dogs from Medellín, our research group reported the presence of CDV of the South America-3 lineage and the combination of 549Y, 519R, and 530N; one dog presented 549H (Table 2). All of these cases showed evidence of nervous symptomatology and respiratory disease with fatal outcomes (Espinal, Díaz, and Ruiz-Saenz 2014). The mutation associations in the CDV *H* gene are an area of study that should be extended to the complete genome to explain the pathogenesis in the various susceptible species. As a model, measles virus tolerates multiple mutations in the genes that express the membrane proteins which interact with cellular

receptors. However, mutations in intergenic regions and in the 5' and 3' UTRs can affect the virulence and pathogenicity of field strains (Beaty and Lee 2016), as has been described for CDV in the intergenic region of the *M* and *F* genes (Anderson and von Messling 2008), showing the importance of conducting these studies in the complete genome.

H **gene vs. full genome analysis**

Although in this review the molecular markers in the protein H of CDV have been described that explain the jump of the barrier of species, pathogenicity and evasion of the immune response, there is still controversy about which mutations are determinants in strains of wild fauna, moreover, the epistatic interactions (compensatory substitutions present in a site that occur as a consequence of a substitution in another site) (Ke et al., 2015) in the complete genome are not known, which explain these three characteristics of the wild strains. the most interesting point to be able to explore multiple mechanisms of viral evolution such as sites or epitopes under selection, sites under homologous recombination, in addition to characteristics such as glycosylation sites and evolutionary rates. An example of the importance of studying the complete genome are the studies carried out on strains of CDV adapted to humans (Table 3) and wildlife that not only describe mutations in H but in all genes (Otsuki, Nakatsu, et al. Nikolin et al., 2016), likewise initial approximations of evolutionary studies speak of homologous recombination in P, H and L genes between field strains and vaccine strains (Yuan et al., 2017). However, it is necessary to obtain more sequences of complete genomes of all circulating lineages worldwide to perform phylogeny, search for selection sites, glycosylation in other proteins such as F to explain vaccine failures and virulence.

Conclusion

The *H* gene is the basis for the phylogenetic classification of CDV due to the high evolutionary pressure to which the protein H is subjected by the host immune system. The variability of the protein H has also allowed researchers to infer key substitutions that may facilitate the inter-species jump. The study of CDV complete genome would enable us to explain in greater detail the evolution of the virus and to establish the mutation/evolutionary rates, glycosylation sites, and homologous recombination points that would contribute to explain the inter-species jump, emerging zoonosis, and vaccine failure of CDV.

Conflict of Interests

The authors of this study have no personal or financial conflicts that may inappropriately influence the content of this document.

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5.CAPÍTULO II

En este capítulo se presenta el trabajo desarrollado para dar cumplimiento del primer objetivo específico de la tesis:

1. Comparar los genotipos de CDV circulantes en Colombia y Ecuador.

Sometido en: Veterinary Microbiology

Phylogenetic evidence of the intercontinental circulation of a CDV lineage in the Americas

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Abstract

Canine Distemper Virus (CDV) is the cause of a multisystem disease in domestic dogs and wild animals, infecting more than 20 carnivores and non-carnivores families, even infecting human cell lines in *in vitro* conditions. The phylogenetic classification based on the Hemagglutinin gene includes 17 lineages with a phylogeographic distribution pattern of distribution. In Medellín (Colombia), circulates the lineage South America 3, which is considered endemic. Phylogenetic studies conducted in Ecuador using the fragment coding for the Fusion protein signal peptide (Fsp), characterized a new strain belonging to a different lineage. To understand the distribution of Lineage South America-3 in the north area of the South American continent, we characterize the CDV from three Colombian cities (Medellín, Bucaramanga and Bogotá). By using phylogenetic analysis of the Hemagglutinin gene and the Fsp region we confirmed the circulation of CDV South America-3 in different areas of Colombia. We also described for the first time the circulation of a new lineage in Medellín presenting a monophyletic group with strains previously characterized in dogs in Ecuador and in wildlife and domestic dogs United States, which we proposed to be named South America/North America-4 lineage due its intercontinental distribution. In conclusion, our results indicate that there are at least four different CDV lineages circulating in domestic dogs in South America. The Europe/South America-1 lineage circulating in Brazil, Uruguay and Argentina; the South America 2 lineage restricted to Argentina; the South America 3, only reported in Colombia and finally an intercontinental lineage present in Colombia, Ecuador and the United States denoted as “South America/North America -4 lineage”.

Keywords:

Genotype, fusion (F) gene, hemagglutinin (H) gene, phylogeny, wild-type isolates

1. Introduction

Canine distemper virus (CDV) belongs to the *Paramixoviridae* family, genus *Morbillivirus*, which includes viruses with epidemiological relevance in human and animal populations (de

Vries et al., 2015). CDV is the etiological agent of a highly prevalent viral infectious disease of domestic and wild carnivores, posing a conservation threat to endangered species around the world (Martinez-Gutierrez and Ruiz-Saenz 2016; Mccarthy, Shaw, and Goodman 2007).

Clinical symptoms in all the species affected are influenced by strain virulence, environmental conditions, host age and immune status. The gastrointestinal tract, respiratory and nervous systems are the most affected in all species (Budaszewski da Fontoura and von Messling 2016; Loots et al. 2017). Clinical signs in CDV infected dogs includes onset of a cutaneous rash, serous nasal and ocular discharge, conjunctivitis and anorexia, followed by gastrointestinal and respiratory signs, which are often complicated by secondary bacterial infections and neurological disorders (Deemd et al. 2000; Beineke, Baumgärtner, and Wohlsein 2015).

From the six encoded viral proteins, the hemagglutinin (H) and fusion (F) glycoproteins are responsible for virus attachment and fusion process to the host cells (B. Sawatsky and von Messling 2010). The H protein is of principal importance as it determines viral tropism *in vivo* and *in vitro* and determines the host-specific immunity (Iwatsuki et al. 2000)

Due to external pressures on the immune system of the host, the H gene displays the higher genetic variability when compared to the other CDV genes (Iwatsuki et al. 2000), which makes it suitable for lineage identification and phylogenetic analysis. Moreover, it has been established that a short region of the F gene that encodes the signal peptide of the F protein (Fsp) is extremely variable suggesting that this region could be also an useful marker for evolutionary studies as it allows for straightforward identification of CDV lineages (Sarute et al. 2013).

Phylogenetic studies based on the complete sequence of the H gene or the Fsp-coding region of several CDV viruses around the world have revealed a geographical pattern of genetic diversity. According to this pattern, there are multiple distinct lineages/genotypes most of them following a geographical pattern of distribution known as America 1 (includes the

commercially available vaccines), America 2-5, Artic-like, Asia 1-4, Africa 1 and 2, European Wildlife, Europe/South America 1, South America 2 and 3 (Blixenkrone-Möller et al. 1992; Haas et al. 1996; Iwatsuki et al. 2000; Deemd et al. 2000; Woma et al. 2010; Panzera et al. 2012; Budaszewski et al. 2014; Espinal, Díaz, and Ruiz-Saenz 2014; Riley and Wilkes 2015)

Although immunization with attenuated vaccines have been largely used to prevent the disease, it has been hypothesized that the strong genetic diversity and wide variability of H gene could alter the antigenic profile of these new genetic variants when compared to vaccine strain affecting specific sites on the H protein that are related with immune neutralization (Blixenkrone-Møller et al. 1993; Iwatsuki et al. 2000; Martella et al. 2006; Budaszewski da Fontoura et al. 2016). In fact, recent analysis has suggested the need to develop an updated CDV vaccine due to the difference in cross-neutralization assays revealing wide antigenic differences among the CDV wild-type isolates and the vaccine strains currently used in the U.S. (Anis, Holford, et al. 2018).

Different publications have shown a worldwide increase in the incidence of the disease, even in vaccinated dog populations (Decaro et al. 2004; Budaszewski et al. 2014; Espinal, Díaz, and Ruiz-Saenz 2014; Romanutti et al. 2016) and due to most CDV commercial vaccines are formulated with strains belonging to the America-1 lineage (Riley and Wilkes 2015); it could be possible that antigenic differences could explain the worldwide increase in the incidence of the disease even in vaccinated dogs.

It has been suggested that the South American continent has one of the highest variabilities of the world (Panzera et al. 2014). Phylogenetic analyses based on CDV H gene from South America have been performed to establish the evolutionary patterns of the virus in the region revealing multiple circulating lineages of CDV with different prevalence. In Brazil, Uruguay and Argentina the most prevalent lineage belongs to the Europe/South America 1 lineage (Panzera et al. 2012; Budaszewski et al. 2014); the second one, known as South America 2,

is restricted to Argentinian canine populations and appears to be related with strains isolated form wild carnivore species in Europe (Calderon et al. 2007).

In the northern part of South America, there has been reported the circulation of different lineages; In Colombia, by using complete H gene sequencing there has been reported a third lineage (South America 3) that causes diseases even in vaccinated dogs (Espinal, Díaz, and Ruiz-Saenz 2014). The same year, a possible different CDV lineage was described based on the analysis of the Fsp-coding region of Ecuadorian strains (Sarute et al. 2014). Due to the difference in methodologies used, complete H gene sequences are therefore not comparable with the Fsp-coding region sequences available from this region of the continent, it could be possible a genetic relation between CDV from those Countries because they have similar geographic distribution in northern region of South America.

To determine the genetic diversity of CDV from Colombia in relation to other regions of South America, we analyzed the genetic diversity within the Fsp-coding region and H gene of CDV strains currently circulating in different regions of Colombia and compared it with America and worldwide circulating wild-type CDV and vaccine strains.

2. Materials and Methods

2.1. Ethical considerations

This study was approved by the Ethics Committee for Animal Experimentation of the Universidad Cooperativa de Colombia in Bucaramanga. Dog owners signed informed consent forms approved by the ethics committee. Additionally, the authors declare that the implementation of this work followed all scientific, technical and administrative rules for animal research.

2.2 Clinical specimens and vaccine strains

Total of 86 clinical samples from dogs exhibiting signs suggestive of CDV where obtained from three main Colombian Provinces (Figure 1). Forty-eight samples were taken from Bogotá D.C. from January 2014 to June 2015, eleven samples were taken from Bucaramanga from June 2016 to November 2016 and twenty-seven Samples were taken from Medellín from May to September 2017. Samples were taken in different veterinary hospitals and basic data were gathered at the time of sampling including dog's age, sex, breed, vaccination status, municipality and/or neighborhood of origin, and clinical signs. Clinical specimens included serum and ocular discharge. Commercially available vaccines were used as positive controls for RT-PCR reactions.

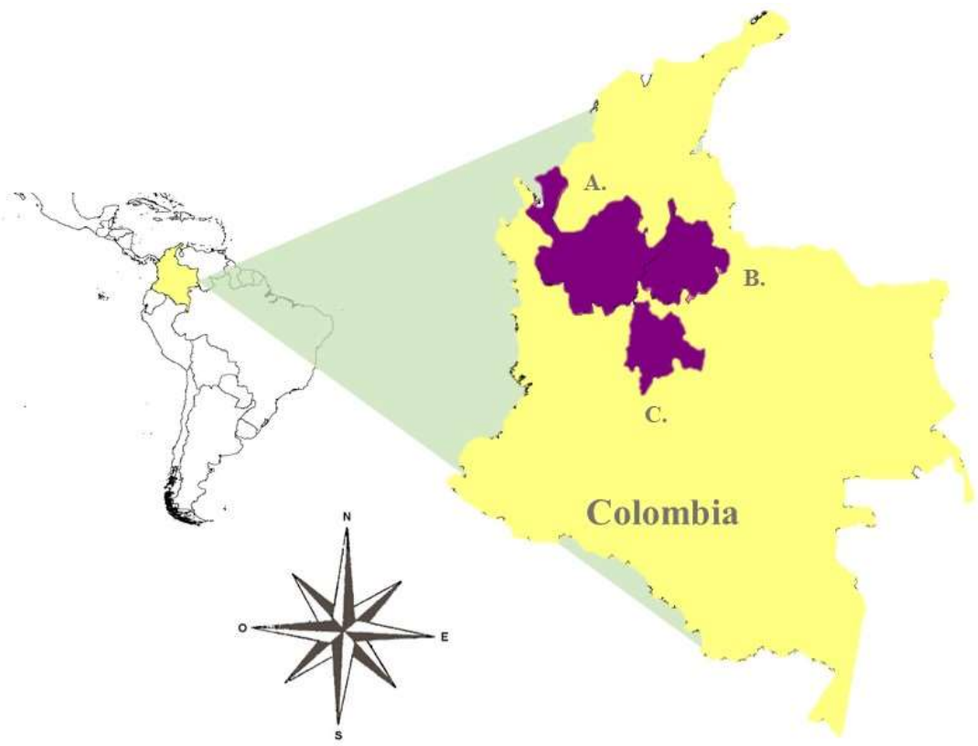

Figure 1. Geographical location of sampling sites in Colombia. Purple area denotes the Medellín (A), Bucaramanga (B) and Bogotá (C) cities. The map was created using the DIVA - GIS software version 7.5.0 for Windows™. See the main text for references.

2.3 RNA extraction

Total RNA was extracted from 140 µl of supernatant of ocular discharge, serum, and four commercial CDV vaccines using the QIAamp Viral RNA (QIAGEN®, Hilden, Germany) Mini Spin procedure according to the manufacturer's instructions. RNA quality and quantity

was determined by spectrophotometric analysis with a NanoDrop™ One UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA), and RNA aliquots were stored at -80ºC until used.

2.4 Complementary DNA (cDNA) synthesis

Synthesis of cDNA was performed using the RevertAid™ Premium First Strand cDNA Synthesis Kit (Thermo Scientific®, Glen Burnie, MD) according to the manufacturer´s instructions. Briefly, a denaturation mix consisting of 1 µl (100 pmol/µl) of random hexamers, 1 µl of dNTP Mix (10 mM) and 13 µl (0.02-4.6 µg) of total RNA was initially denatured at 65°C for 5 min and immediately incubated on ice. The RT mix solution consisted in 4 µl of 5X Reverse Transcriptase Buffer and 1 µl of RevertAid™ Premium Enzyme Mix. The RT mix was added to the denaturation mix and reverse transcription was perform in a total volume of 20 µl in a ProFlex™ PCR Thermal Cycler (Applied Biosystems®, Foster city, California, USA) for 10 min at 25°C followed by 30 min at 50ºC; the reaction was terminated by heating at 85°C for 5 min. The reaction product was stored at -80ºC until used.

2.5 PCR and sequencing

cDNAs from clinical specimens were screened by PCR of the phosphoprotein (P) gene using the Maxima Hot Start PCR Master Mix (2X) (Thermo Scientific®) reagent kit according to the manufacturer´s instructions. Detection of viral cDNA was performed using morbillivirus universal primers (Barrett et al., 1993), that amplify a 429 bp fragment of the phosphoprotein gene. Four µl of cDNA was added to a PCR reaction mix which consisted of 25 µl of Maxima Hot Start PCR Master Mix (2X), 15 µl of nuclease-free water and 3 µl (10 µM) of each of the forward and reverse primers. PCR was performed on a ProFlex™ PCR Thermal Cycler (Applied Biosystems®) under the following conditions: initial denaturation at 95ºC for 4 min, followed by 35 cycles of denaturation at 95ºC for 30 sec, annealing at 50.8ºC for 30 sec, extension at 72ºC for 1 min, and a final extension at 72ºC for 5 min. Ultrapure water was used as a negative control and cDNA from one of the vaccines as positive control.

In all samples that tested positive for the P gene, the full length H gene and the Fsp-coding region were amplified by using the Maxima Hot Start PCR Master Mix kit according to the manufacturer´s instructions. Detection of the H gene was performed using primers CDVff1 and HS2 (Lan et al. 2006) that amplify a 2099 bp fragment of the CDV genome, comprising the H gene and flanking regions at both ends. Amplification of Fsp-coding region were performed by using the primers CDV-F4854 and CDV-R5535 (Sarute et al. 2013) or the primers F5/R5 (Riley and Wilkes 2015) flanking the Fsp-coding region. In all cases, Four µl of cDNA was added to a PCR reaction mix which consisted of 25 µl of Maxima Hot Start PCR Master Mix (2X), 15 µl of nuclease-free water and 3 µl (10 µM) of each of the primers (Table 1). PCR was performed on a ProFlex™ PCR Thermal Cycler (Applied Biosystems®) under the following conditions: initial denaturation at 95ºC for 4 min, followed by 35 cycles of denaturation at 95ºC for 30 sec, annealing for 30 sec, extension at 72ºC for 2 min, and a final extension at 72ºC for 10 min. The annealing temperatures for H gen was of 48.2°C, and for the Fsp-coding region were 50.8°Cfor the F5/R5 primers and 58°C for the CDV-F4854/R5535 primers.

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

Oligonucleotide label	Oligonucleotide sequence	Genomic position*	Reference
	Amplification of P gene		
CDV Universal (forward)	ATGTTTATGATCACAGCGGT	2132–2151	Daly et al, 2006
CDV Universal (reverse)	ATTGGGTTGCACCACTTGTC	2541–2560	Daly et al, 2006
	Amplification and sequencing of H gene		
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
	Amplification and sequencing of Fsp-coding region		
F5 (forward)	TGTTACCCGCTCATGGAGAT	4272–4292	Riley and Wilkes, 2015
R5 (reverse)	CCAAGTACTGGTGACTGGGTCT	5411–5433	Riley and Wilkes, 2015
CDV-F4854 (Forward)	TCCAGGACATAGCAAGCCAACA	4854 - 4875	Sarute et al., 2013
CDV-R5535 (Reverse)	GGTTGATTGGTTCGAGGACTGAA	5513 - 5535	Sarute et al., 2013

*** Reference genome AF164967 (A75/17)**

Following PCR, 5 µl of amplicons were analyzed by gel electrophoresis in a 1.5% agarose gel (AGAROSE I™, Amresco, Solon, OH, USA) at 110 V for 60 min. The Gels were stained using EZ-VISION™ dye (Amresco1 Solon, OH, USA) and viewed by trans-illumination with UV light using the Molecular Imager® GelDoc TM XR+ System with the image acquisition software ImageLab™ (Bio-Rad, Hercules, CA, USA). Amplification product sizes were estimated using a molecular weight ladder from 100 to 3000 bp (GeneRuler™ 100 bp Plus DNA Ladder, Thermo Scientific®)

PCR amplicons of H gene and Fsp-coding region were submitted for purification and sequencing to Macrogen Inc., (Macrogen Inc., Seoul, Korea). An additional set of eight primers published elsewhere for the H gene were used for sequencing (Pardo, Johnson, and Kleiboeker 2005; Lan et al. 2006) (Table 1) using an ABI3711™ automatic sequencer (Macrogen™).

2.6 Phylogenetic analysis

Sequence data were assembled and edited using the SeqMan program (DNAStar Lasergene™ V15.0 software package, Madison, Wisconsin, USA). Nucleotide BLAST (Basic Local Alignment Search Tool) was used to explore sequence similarity of Colombian CDV strains with all the available sequences of CDV in the NCBI nucleotide databases. For the H gene, a total length of 1824 nucleotide and deduced amino acid sequences were obtained only from dogs belonging to Medellín and Bucaramanga Cities and for the Fsp-coding region (405 nucleotides and its deduced aminoacid sequence) samples were obtained from all three studied cities (Bogotá DC, Medellín and Bucaramanga). Phylogenetic analyses were carry out with at least ten sequences by each reported lineage and vaccine strains from different geographical regions by using MEGA™ 7 (Kumar, Stecher, and Tamura 2016) and the Muscle algorithm and the nucleotide and amino acid differences were assessed as uncorrected (p) distances.

The phylogenetic relationships based on the nucleotide alignment of the complete H gene sequences were inferred using distance-based (neighbor-joining) and character-based

(maximum likelihood, Bayesian) approaches implemented in MEGA™ 7 and MrBayes 3.2.6 (Ronquist et al., 2012) software, respectively. The best fit model for nucleotide substitution was identified by MEGA™ 7 as Tamura 3 parameters gamma distribution rate heterogeneity (T92+ G) according to the Bayesian information criterion (BIC) for the H gene and the Hasegawa-Kishino-Yano with gamma distribution (G) (HKY+G) for the Fsp-coding region. 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 T92+G model is not implemented in MrBayes 3.2.6. For this method, two parallel analyses were run for 1,000,000 generations with a 25% burn-in period. The convergence of the MCMC chains was assessed by the standard deviation of split frequencies, which fall below 0.01. America 1 lineage was used as outgroup to root the phylogenetic trees. The consensus trees where edited in Figtree software, version 1.4 (Rambaut 2018).

2.7 Amino acid analysis of the H protein and the Fsp Peptide

The deduced amino acid sequences of the H protein of the Colombian wild-type CDV strains (607 aa) and the Fsp peptide (135 aa) where aligned with multiple CDV protein sequences from different geographical regions using MEGA™ 7 to explore their amino acid profile and the potential differences with vaccine and wild-type strains of the already known CDV lineages. Prediction of potential N-linked glycosylation sites was performed with NetNGlyc 1.0 (Gupta, Jung, and Brunak 2017)

2.8 Sites under positive selection

To identify amino acid sites in the CDV H protein and Fsp peptide under positive selection, the ratio of non-synonymous (dN) to synonymous (dS) substitutions was calculated by using ML phylogenetic reconstruction and the general reversible nucleotide substitution model available through the Datamonkey web server. To detect non-neutral selection the Fast Unconstrained Bayesian Approximation (FUBAR), within the HyPhy software package were implemented in Datamonkey (Murrell et al. 2013) . The range of significance for the posterior probability was 0-1. Generally, posterior probabilities > 0.9 are strongly suggestive

of positive selection. Finally, Bayes factor = 50 was used to estimate the rates ofdN and dS within each codon. The values dN/dS > 1, dN/dS = 1 and dN/dS < 1 were used to define the positive selection (adaptive molecular evolution), neutral mutations, and negative selection (purifying selection), respectively.

2.9 H gene and Fsp phylogeography

The mean substitution rate (substitutions per site per year), the time to the most recent common ancestor (tMRCA), the geographic origin, and the overall spatial dynamics of the major CDV clades were inferred using a Bayesian approach of the Monte Carlo Markov Chain (MCMC) implemented in the BEAUti / BEAST v1.8.4 package. (Drummond et al. 2012) The analysis was implemented using a strict molecular clock with a constant population size, 3E07 generations were run in order to ensure the effective population size (ESS) greater than 200 for the evaluated parameters using the Tracer v1.7 program (Drummond et al. 2012). The initial 10% of the MCMC was eliminated, which corresponds to low probability states at the beginning of the chain. The tree of maximum credibility of the MCC clades, was built with TreeAnnotator and visualized with FigTree v1.4.3 (Rambaut 2007).

3. Results

3.1 Detection of P gene and clinical features

A fragment of 429 bp of the phosphoprotein gene was detected in 68 (79.1%) clinical specimens of the 86 dogs sampled. Thus, by regions we obtained CDV presentation frequencies of 9% (1/11) in Bucaramanga, 100% (48/48) in Bogota and 70% (19/27) in Medellin. 44.1% of the CDV positive animals were male and 55.9% were female. Young dogs from one to six months old were the most affected (46.9%), although disease presentation in dogs older than 12 months was also observed (21.9%). Concerning clinical manifestations in affected dogs, nervous and respiratory diseases accounted for 25% of the cases, close followed by the presentation of respiratory signs alone (21.4%). An equal

proportion (17,9%) of clinical ill dogs has presentation of tegumentary/respiratory/nervous signs or only respiratory/digestive disease.

3.2 Sequence analysis of the H gene and the Fsp-coding region

We only amplified and sequenced a fragment of 2099 bp for H gene in six clinical specimens. By this, the 405 bp from the Fsp-coding region was assessed, getting positive amplifications and sequencing in total 23 clinical samples. Information regarding age, gender, breed, vaccination status, clinical signs and outcome, as well as accession numbers from H gene positive samples is summarized in Table 2.

Table 2. Clinical features of Colombian dogs infected with canine distemper virus (CDV)

Code	Sample[a]	Sex[b]	Age[c]	Clinical signs[d]	Vaccination status	Outcome	Gene	Region
MDE 2a/CO/2017	NS	M	2 Y	R, GI	Unknown	Euthanized	H,F	RIONEGRO
MDE 2aP/CO/2017	CS	F	6M	R,N	Unknown	Euthanized	H,F	RIONEGRO
MDE 1aM/CO/2017	CS	M	8Y	R	Incomplete vacination	Euthanized	H,F	RIONEGRO
MDE 13b/CO/2017	CS	M	7Y	R,N	Not vaccinated	Euthanized	H,F	MEDELLIN
MDE 16a/CO/2017	CS	F	9M	N	Not vaccinated	Euthanized	H,F	MEDELLIN
MDE 18 a/CO/2017	CS	M	7M	R,N	Incomplete vacination	Euthanized	H,F	MEDELLIN
BUCA 12/CO/2016	CS	F	3M	R,N	Incomplete	Died	F	BUCARAMANGA
MDE 19a/CO/2017	CS	F	5M	R	Incomplete	Unknown	F	MEDELLIN
MDE 9a/CO/2017	CS	F	7Y	R,N	Not vaccinated	Euthanized	F	MEDELLIN

CM-14-027	BLOOD	Unknown	1M	R,GI,N	Unknown	Unknown	F	BOGOTÁ
CM-14-160	URINE	Unknown	2M	R,GI,N	Unknown	Unknown	F	BOGOTÁ
CM-15-001	URINE	Unknown	Unknown	R	Unknown	Unknown	F	BOGOTÁ
CM-15-061	URINE	Unknown	8M	R	Unknown	Unknown	F	BOGOTÁ
CM-15-089	BLOOD	Unknown	4M	WITHOUT SIGNS	Unknown	Unknown	F	BOGOTÁ
CM-15-079	BLOOD	Unknown	4M	WITHOUT SIGNS	Unknown	Unknown	F	BOGOTÁ
CM-15-018	URINE	Unknown	3M	Unknown	Unknown	Unknown	F	BOGOTÁ
CM-15-052	URINE	Unknown	14M	Unknown	Unknown	Unknown	F*	BOGOTÁ
CM-15-061	URINE	Unknown	8M	R	Unknown	Unknown	F*	BOGOTÁ
CM-15-066	URINE	Unknown	6M	R,GI,N	Unknown	Unknown	F	BOGOTÁ
CM-15-069	BLOOD	Unknown	5M	WITHOUT SIGNS	Unknown	Unknown	F	BOGOTÁ
CM-15-078	BLOOD	Unknown	4M	WITHOUT SIGNS	Unknown	Unknown	F	BOGOTÁ
CM-15-135	NS	Unknown	4Y	R	Unknown	Unknown	F*	BOGOTÁ
CM-15-171	BLOOD	Unknown	3M	GI,T	Unknown	Unknown	F	BOGOTÁ

[a]S: Serum; CS: Conjunctival swab – [b] F: female; M: male – [c] M: months; Y: Years – [d] R: Respiratory; GI: Gastrointestinal; O: Ocular; N: Neurological; T: Tegumentary. * Sequences don´t include in the phylogenetic analysis.

The Fsp-coding region sequences of the strains Medellín D/Mde_19a/CO/2017, D/Mde_2aP/CO/2017, D/Mde_13b/CO/2017 and D/Mde_16a/CO/2017 were 100% identical. Likewise, Bogota sequences D/Bog-4/CO/2015, D/Bog-5/CO/2015 and D/Bog-6/CO/2015 were 100% identical between them, by this, only one of those sequences (D/Bog-4/CO/2015) where included with the rest of the Colombian strains in the phylogenetic analysis. Vaccines used as positive controls in the PCR reactions were also sequenced.

Colombian H sequences submitted to analysis displayed high identity to each other (93.5%-99.9% nt; 93%-99.9% aa) and the overall mean distance was 0.039. The alignment of the H gene of Colombian CDV and the Onderstepoort vaccine strain (AF378705) showed an identity that varied from 89.6%-91.1% at aa level and 90.8%-91.8 % at nt. As expected, a higher in variability in the Fsp-coding region was found (81.39%-99.01% nt – 63.91%-97.7% aa) and the overall mean distance was 0.1014. Moreover, the Fsp-coding region Colombian sequences displayed very low identity with Onderstepoort vaccine strain (80.9%-83.6% nt and 57.6%-67.4% aa)

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 a similar topology. Phylogenetic tree of H gene identified 16 lineages with a defined geographical distribution pattern (the lineage Asia-3 is grouped with the strains of the lineage America-1); while the Fsp tree only identified 15 lineages mainly because there are no available sequences for the Fsp for the Europe Wildlife lineage.

Interestingly, we showed that Colombian CDV sequences cluster in two different branches in both Fsp segment and H gene trees (Figures 2 and 3); a group of Colombian Fsp-coding clusters in the same clade with Ecuadorian strains (Figure 2) and interestingly it clusters with the two recently reported North America-4 Lineage sequences (Riley and Wilkes, 2015) (97.1% identity). Other Colombian CDV sequences cluster with the South America-3 lineage previously reported in Colombia (Figure 3). Unfortunately, no Ecuadorian H sequences has been reported up to date.

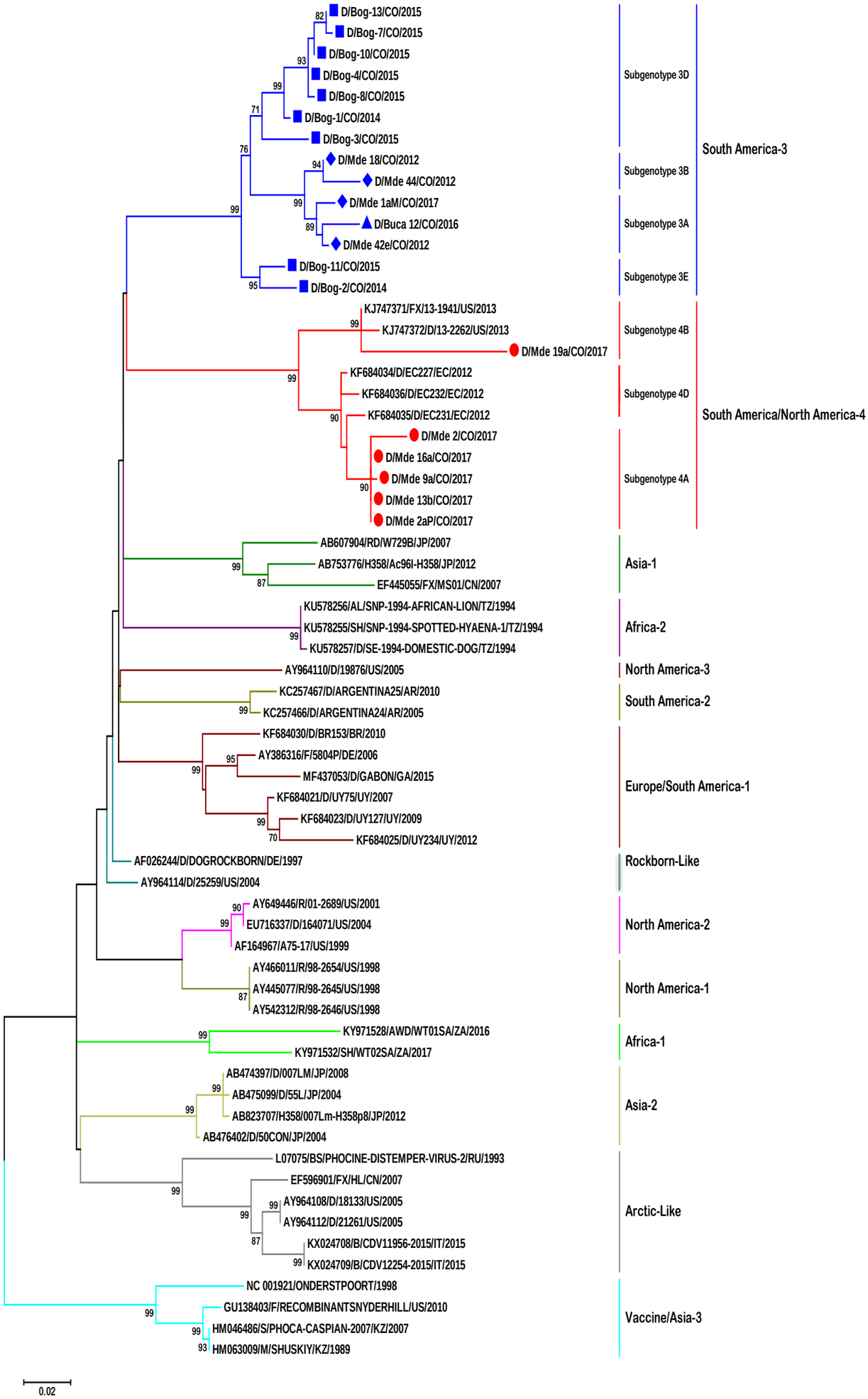
D/Bog-13/CO/2015
D/Bog-7/CO/2015
D/Bog-10/CO/2015
D/Bog-4/CO/2015
D/Bog-8/CO/2015
D/Bog-1/CO/2014
D/Bog-3/CO/2015
D/Mde 18/CO/2012
D/Mde 44/CO/2012
D/Mde 1aM/CO/2017
D/Buca 12/CO/2016
D/Mde 42e/CO/2012
D/Bog-11/CO/2015
D/Bog-2/CO/2014
KJ747371/FX/13-1941/US/2013
KJ747372/D/13-2262/US/2013
D/Mde 19a/CO/2017
KF684034/D/EC227/EC/2012
KF684036/D/EC232/EC/2012
KF684035/D/EC231/EC/2012
D/Mde 2/CO/2017
D/Mde 16a/CO/2017
D/Mde 9a/CO/2017
D/Mde 13b/CO/2017
D/Mde 2aP/CO/2017
AB607904/RD/W729B/JP/2007
AB753776/H358/Ac96I-H358/JP/2012
EF445055/FX/MS01/CN/2007
KU578256/AL/SNP-1994-AFRICAN-LION/TZ/1994
KU578255/SH/SNP-1994-SPOTTED-HYAENA-1/TZ/1994
KU578257/D/SE-1994-DOMESTIC-DOG/TZ/1994
AY964110/D/19876/US/2005
KC257467/D/ARGENTINA25/AR/2010
KC257466/D/ARGENTINA24/AR/2005
KF684030/D/BR153/BR/2010
AY386316/F/5804P/DE/2006
MF437053/D/GABON/GA/2015
KF684021/D/UY75/UY/2007
KF684023/D/UY127/UY/2009
KF684025/D/UY234/UY/2012
AF026244/D/DOGROCKBORN/DE/1997
AY964114/D/25259/US/2004
AY649446/R/01-2689/US/2001
EU716337/D/164071/US/2004
AF164967/A75-17/US/1999
AY466011/R/98-2654/US/1998
AY445077/R/98-2645/US/1998
AY542312/R/98-2646/US/1998
KY971528/AWD/WT01SA/ZA/2016
KY971532/SH/WT02SA/ZA/2017
AB474397/D/007LM/JP/2008
AB475099/D/55L/JP/2004
AB823707/H358/007Lm-H358p8/JP/2012
AB476402/D/50CON/JP/2004
L07075/BS/PHOCINE-DISTEMPER-VIRUS-2/RU/1993
EF596901/FX/HL/CN/2007
AY964108/D/18133/US/2005
AY964112/D/21261/US/2005
KX024708/B/CDV11956-2015/IT/2015
KX024709/B/CDV12254-2015/IT/2015
NC 001921/ONDERSTPOORT/1998
GU138403/F/RECOMBINANTSNYDERHILL/US/2010
HM046486/S/PHOCA-CASPIAN-2007/KZ/2007
HM063009/M/SHUSKIY/KZ/1989
Subgenotype 3D
Subgenotype 3B
Subgenotype 3A
Subgenotype 3E
South America-3
Subgenotype 4B
Subgenotype 4D
Subgenotype 4A
South America/North America-4
Asia-1
Africa-2
North America-3
South America-2
Europe/South America-1
Rockborn-Like
North America-2
North America-1
Africa-1
Asia-2
Arctic-Like
Vaccine/Asia-3
0.02

Figure 2. Phylogenetic relationships between 64 CDV strains based on Fsp fragment gene sequences. The phylogenetic tree was inferred by the Maximum Likelihood method using 1000 replicates. GenBank accession numbers, the species from which each isolate was obtained, name of the strain, 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: AL: African lion (*Panthera leo*), AWD: African wild dog (*Lycaon pictus*), B: Badger (*Meles meles)*, BS: Baikal seal (*Pusa sibirica*), D: Dog (*Canis lupus familiaris*), F: Ferret (*Mustela putorius furo*), FX: Fox (*Vulpes urocyon*), H358: Human lung cells, M: Mink (*Neovison vison*), R: Raccoon (*Procyon lotor)*, RD: Raccoon dog (*Nyctereutes procyonoides)*, S: Seal (*Phoca vitulina*), SH: Spotted hyaena (*Crocuta crocuta*). Abbreviations for countries: AR: Argentina, BR: Brazil, CN: China, CO: Colombia, DE: Germany, EC: Ecuador, GA: Gabon, IT: Italy, JP: Japan, KZ: Kazakhstan, RU: Russia, TZ: Tanzania, US: United States, UY: Uruguay, ZA: South Africa.

Keeping in mind that Colombian viruses showed high variability at nucleotide and amino acid differences, we analyze the identity of Colombian CDV H sequences and the previously reported CDV lineages (Table 3). By the analysis of the Uncorrected (p) distances, we found that Colombian and Ecuadorian CDV strains that we named South America-4 lineage had less than 4% aminoacid difference with the previously reported North America-4 Lineage. According to this, we propose that this lineage should be designated as "South America/North America-4" due its intercontinental distribution. Remarkable, we observed a high variation (10% approximately) between Colombian CDV lineages and North America-1 lineage that includes most of the commercial vaccine strains (Table 3).

3.3 CDV Subgenotypes analysis

In the CDV subgenotypes analysis based on Measles criteria (H amino acid identity of 98% and Bootstrap> 70%), we found at least three subgenotypes in the South America-3 Lineage. Subgenotype A: strains 13-CO-12; 19-CO-2012; 26-CO-12 and 1aM-CO-2017 (amino acid variation between 0.2-0.06%), subgenotype B: strains 18-CO-2012, 40-CO-2012 and 44-CO-

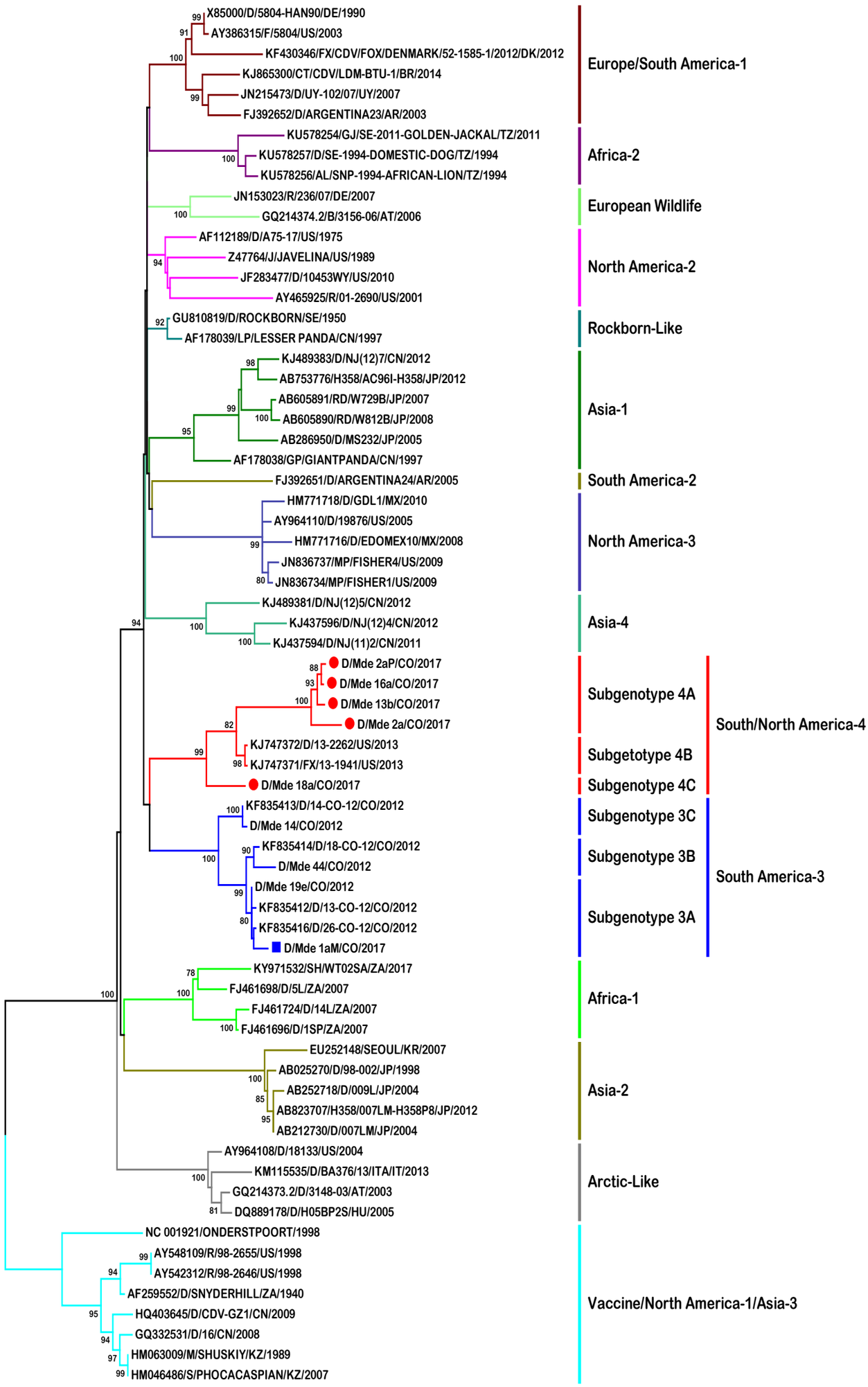
X85000/D/5804-HAN90/DE/1990
AY386315/F/5804/US/2003
KF430346/FX/CDV/FOX/DENMARK/52-1585-1/2012/DK/2012
KJ865300/CT/CDV/LDM-BTU-1/BR/2014
JN215473/D/UY-102/07/UY/2007
FJ392652/D/ARGENTINA23/AR/2003
Europe/South America-1
KU578254/GJ/SE-2011-GOLDEN-JACKAL/TZ/2011
KU578257/D/SE-1994-DOMESTIC-DOG/TZ/1994
KU578256/AL/SNP-1994-AFRICAN-LION/TZ/1994
Africa-2
JN153023/R/236/07/DE/2007
GQ214374.2/B/3156-06/AT/2006
European Wildlife
AF112189/D/A75-17/US/1975
Z47764/J/JAVELINA/US/1989
JF283477/D/10453WY/US/2010
AY465925/R/01-2690/US/2001
North America-2
GU810819/D/ROCKBORN/SE/1950
AF178039/LP/LESSER PANDA/CN/1997
Rockborn-Like
KJ489383/D/NJ(12)7/CN/2012
AB753776/H358/AC96I-H358/JP/2012
AB605891/RD/W729B/JP/2007
AB605890/RD/W812B/JP/2008
AB286950/D/MS232/JP/2005
AF178038/GP/GIANTPANDA/CN/1997
Asia-1
FJ392651/D/ARGENTINA24/AR/2005
South America-2
HM771718/D/GDL1/MX/2010
AY964110/D/19876/US/2005
HM771716/D/EDOMEX10/MX/2008
JN836737/MP/FISHER4/US/2009
JN836734/MP/FISHER1/US/2009
North America-3
KJ489381/D/NJ(12)5/CN/2012
KJ437596/D/NJ(12)4/CN/2012
KJ437594/D/NJ(11)2/CN/2011
Asia-4
D/Mde 2aP/CO/2017
D/Mde 16a/CO/2017
D/Mde 13b/CO/2017
D/Mde 2a/CO/2017
Subgenotype 4A
KJ747372/D/13-2262/US/2013
KJ747371/FX/13-1941/US/2013
Subgetotype 4B
D/Mde 18a/CO/2017
Subgenotype 4C
South/North America-4
KF835413/D/14-CO-12/CO/2012
D/Mde 14/CO/2012
Subgenotype 3C
KF835414/D/18-CO-12/CO/2012
D/Mde 44/CO/2012
Subgenotype 3B
D/Mde 19e/CO/2012
KF835412/D/13-CO-12/CO/2012
KF835416/D/26-CO-12/CO/2012
D/Mde 1aM/CO/2017
Subgenotype 3A
South America-3
KY971532/SH/WT02SA/ZA/2017
FJ461698/D/5L/ZA/2007
FJ461724/D/14L/ZA/2007
FJ461696/D/1SP/ZA/2007
Africa-1
EU252148/SEOUL/KR/2007
AB025270/D/98-002/JP/1998
AB252718/D/009L/JP/2004
AB823707/H358/007LM-H358P8/JP/2012
AB212730/D/007LM/JP/2004
Asia-2
AY964108/D/18133/US/2004
KM115535/D/BA376/13/ITA/IT/2013
GQ214373.2/D/3148-03/AT/2003
DQ889178/D/H05BP2S/HU/2005
Arctic-Like
NC 001921/ONDERSTPOORT/1998
AY548109/R/98-2655/US/1998
AY542312/R/98-2646/US/1998
AF259552/D/SNYDERHILL/ZA/1940
HQ403645/D/CDV-GZ1/CN/2009
GQ332531/D/16/CN/2008
HM063009/M/SHUSKIY/KZ/1989
HM046486/S/PHOCACASPIAN/KZ/2007
Vaccine/North America-1/Asia-3

Figure 3. Phylogenetic relationships between 68 CDV strains based on H gene sequences. The phylogenetic tree was inferred by the Maximum Likelihood method using 1000 replicates. GenBank accession numbers, the species from which each isolate was obtained, name of the strain, 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: AL: African lion (*Panthera leo*), B: Badger (*Meles meles),* CT: *Cerdocyon thous*, D: Dog (*Canis lupus familiaris*), F: Ferret (*Mustela putorius furo*), FX: Fox (*Vulpes urocyon*), GJ: Golden jackal (*Canis aureus*), GP: Giant panda (*Ailuropoda melanoleuca*), H358: Human lung cells, J: Javelina (*Tayassu pecari*), LP: Lesser panda (*Ailurus fulgens*), M: Mink (*Neovison vison*), MP: *Martes pennanti*, R: Raccoon (*Procyon lotor),* RD: Raccoon dog (*Nyctereutes procyonoides),* S: Seal (*Phoca vitulina*), SH: Spotted hyaena (*Crocuta crocuta*). Abbreviations for countries: AR: Argentina, AT: Austria,BR: Brazil, CN: China, CO: Colombia, DE: Germany, DK: Denmark, HU: Hungary, IT: Italy, JP: Japan, KR: South Korea, KZ: Kazakhstan, MX: Mexico, SE: Sweden, TZ: Tanzania, US: United States, UY: Uruguay, ZA: South Africa.

2012 (amino acid variation between 0.8-1%), subgenotype C: strain 14-CO-2012 and 39-CO-2012 (amino acid variation 2.2%) (3A to 3C in Figure 3).

Also, within the “South America/North America-4” lineage we found three subgenotypes. Subgenotype A: strains 16a-CO-2017, 2aP / CO-2017, 13b-CO-2017 and 2a-CO-2017 (amino acid variation between 0.2-1.4%); Subgenotype B: strains 13-1941-US-2013 and 13-2262-US-2013 (amino acid variation 2.6%) (American strains) and subgenotype C: strain 18a-CO-2017 (amino acid variation of 4.3%) (4A to 4C in Figure 3).

To evaluate this sub-classification for the Fsp Coding region, we arbitrarily extrapolate this classification and we found five subgenotypes in the South America-3 Lineage (3A to 3E in Figure 2). subgenotype A: strains 42-CO-2012; 1aM-CO-2017 and BUC-12-CO-2016 (amino acid variation between 1.2 -2.5%), subgenotype B: strains 18-CO-2012 and 44-CO-12 (amino acid variation between 2-3.5%), similar results to gene H, and we see two other subgenotypes D: 8 strains of Bogotá 2015 (amino acid variation between 4.7- 5.7%) and subgenotype E: strains BOG2-CO-2014 and BOG11-CO-2015 (amino acid variation between 5.5-5.7%) (strains not characterized with H) .

Likewise, in the Fsp region analysis of the “South America/North America-4” lineage, we observed four well defined subgenotypes (4A to 4D in Figure 2). The subgenotype A: strains 16-CO-2017, 2aP / CO-2017, 13b-CO-2017, 9-CO-2017 and 2-CO-2017 (amino acid variation between 0 -1.5%); Subgenotype B: 13-1941-US-2013, 13-2262-US-2013 and 19-CO-2017 (amino acid variation between 5.5-11.2%) subgenotype D: Ecuadorian strains not characterized with the H gene (amino acid variation 1.5-2 %).

3.4 Amino acid analysis of the H protein

Analysis of the deduced amino acid sequences of the full-length H protein (607 amino acids) in Colombian CDV viruses showed the presence unique substitutions as in the South America-3 (N261S, G488R, T544S) as in South America/North America-4 lineage (E333V, T348K). Remarkable, we found a set of substitutions that are common to both Colombian lineages (T193I, V198I, E333V, S343L, T348K, A365T); however, this substitutions in the South America-3 lineage appears in the new sequences, not in the previously 2012 reported sequences.

Colombian strains showed the same residue of isoleucine at position 506 as vaccine strains belonging to the America 1 lineage. Several substitutions in Colombian strains were also present in other wild-type strains reported in different lineages: S22R (America 2), V41I (European wild life), N128S (Africa), K281R (America 2), G314S (Asia 2 and America 2), I315V (European wild life). All Colombian South America-3 Lineage sequences displayed an asparagine at position 530, a highly variable residue linked to interspecies transmission of the virus (McCarthy et al., 2007). The South America-4 sequences included a Serine at 530 position while North America-4 displayed an Aspartic Acid in such position. The H sequences of the 2017 South America/North America-4 lineage displayed the following unique substitutions: Q5R, L38S, T193I, V198I, V235I, T291M, E333V, H339D, S341L, T348K, F353I

Table 3 Uncorrected distances (p) observed in pairs of amino acid sequences of the F and H genes between CDV lineages

	NA-4	SA-4	SA-3	VAC	SA-2	NA-1	NA-2	NA-3	EU/SA-1	ARC-L	EU-WL	AFR-1	AFR-2	ASIA-1	ASIA-2	ASIA-3	RCK-L	ASIA-4
NA-4		**0,024**	**0,035**	**0,039**	**0,036**	**0,038**	**0,037**	**0,036**	**0,032**	**0,38**	**NA**	**0,038**	**0,037**	**0,035**	**0,038**	**0,041**	**0,033**	**NA**
		0,01	0,018	0,022	0,017	0,021	0,013	0,016	0,015	0,02	0,017	0,017	0,017	0,016	0,02	0,022	0,013	0,017
SA-4	**0,124**		**0,034**	**0,038**	**0,034**	**0,037**	**0,035**	**0,036**	**0,032**	**0,036**	**NA**	**0,036**	**0,036**	**0,034**	**0,036**	**0,039**	**0,031**	**NA**
	0,028		0,018	0,022	0,017	0,021	0,014	0,016	0,016	0,02	0,018	0,017	0,017	0,016	0,02	0,022	0,014	0,017
SA-3	**0,292**	**0,314**		**0,036**	**0,033**	**0,033**	**0,03**	**0,033**	**0,028**	**0,035**	**NA**	**0,035**	**0,033**	**0,031**	**0,034**	**0,038**	**0,028**	**NA**
	0,064	0,078		0,024	0,019	0,023	0,016	0,016	0,016	0,021	0,019	0,019	0,019	0,017	0,021	0,024	0,016	0,018
VAC	**0,371**	**0,405**	**0,367**		**0,037**	**0,033**	**0,037**	**0,038**	**0,035**	**0,037**	**NA**	**0,036**	**0,038**	**0,037**	**0,038**	**0,017**	**0,035**	**NA**
	0,111	0,12	0,132		0,02	0,01	0,02	0,020	0,019	0,022	0,022	0,019	0,021	0,021	0,022	0,012	0,02	0,022
SA-2	**0,257**	**0,262**	**0,249**	**0,332**		**0,035**	**0,033**	**0,035**	**0,029**	**0,036**	**NA**	**0,035**	**0,035**	**0,3**	**0,036**	**0,038**	**0,028**	**NA**
	0,054	0,06	0,076	0,099		0,019	0,014	0,016	0,013	0,019	0,018	0,017	0,018	0,016	0,019	0,021	0,014	0,016

NA-1	**0,284**	**0,324**	**0,255**	**0,228**	**0,239**		**0,026**	**0,036**	**0,032**	**0,035**	**NA**	**0,034**	**0,035**	**0,034**	**0,037**	**0,038**	**0,03**	**NA**
	0,105	0,112	0,127	0,04	0,09		0,019	0,020	0,018	0,021	0,021	0,019	0,021	0,021	0,022	0,009	0,019	0,022
NA-2	**0,246**	**0,268**	**0,206**	**0,321**	**0,194**	**0,104**		**0,033**	**0,028**	**0,035**	**NA**	**0,034**	**0,032**	**0,03**	**0,035**	**0,04**	**0,025**	**NA**
	0,045	0,058	0,069	0,108	0,051	0,01		0,012	0,01	0,017	0,013	0,014	0,014	0,012	0,017	0,02	0,008	0,013
NA-3	**0,261**	**0,285**	**0,251**	**0,313**	**0,228**	**0,231**	**0,201**		**0,03**	**0,037**	**NA**	**0,035**	**0,035**	**0,034**	**0,039**	**0,041**	**0,03**	**NA**
	0,043	0,059	0,056	0,1	0,049	0,094	0,044		0,01	0,018	0,016	0,016	0,016	0,015	0,019	0,021	0,012	0,016
EU/SA-1	**0,242**	**0,263**	**0,235**	**0,337**	**0,205**	**0,242**	**0,176**	**0,216**		**0,034**	**NA**	**0,032**	**0,03**	**0,028**	**0,031**	**0,036**	**0,022**	**NA**
	0,048	0,063	0,059	0,093	0,042	0,088	0,046	0,033		0,017	0,015	0,014	0,015	0,014	0,017	0,019	0,01	0,013
ARC-L	**0,355**	**0,344**	**0,318**	**0,323**	**0,296**	**0,261**	**0,259**	**0,294**	**0,287**		**NA**	**0,034**	**0,035**	**0,036**	**0,033**	**0,038**	**0,032**	**NA**
	0,085	0,099	0,106	0,121	0,081	0,114	0,083	0,078	0,078		0,02	0,018	0,021	0,018	0,017	0,022	0,018	0,019
EU-WL	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**		**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**
	0,063	0,076	0,085	0,118	0,066	0,111	0,058	0,06	0,062	0,094		0,017	0,017	0,016	0,02	0,022	0,014	0,017
AFR-1	**0,343**	**0,351**	**0,3**	**0,325**	**0,267**	**0,25**	**0,254**	**0,291**	**0,273**	**0,288**	**NA**		**0,036**	**0,032**	**0,035**	**0,038**	**0,031**	**NA**

	0,064	0,08	0,084	0,099	0,07	0,093	0,062	0,062	0,06	0,074	0,071		0,018	0,016	0,018	0,02	0,014	0,017
AFR-2	**0,275**	**0,291**	**0,237**	**0,312**	**0,252**	**0,241**	**0,189**	**0,241**	**0,202**	**0,252**	**NA**	**0,316**		**0,033**	**0,037**	**0,04**	**0,029**	**NA**
	0,064	0,079	0,083	0,109	0,056	0,107	0,06	0,061	0,064	0,1	0,073	0,078		0,017	0,021	0,022	0,014	0,018
ASIA-1	**0,274**	**0,302**	**0,245**	**0,343**	**0,215**	**0,256**	**0,204**	**0,254**	**0,223**	**0,322**	**NA**	**0,305**	**0,242**		**0,034**	**0,039**	**0,027**	**NA**
	0,05	0,063	0,068	0,109	0,079	0,106	0,048	0,048	0,049	0,085	0,064	0,064	0,066		0,016	0,022	0,012	0,014
ASIA-2	**0,308**	**0,307**	**0,27**	**0,317**	**0,248**	**0,226**	**0,203**	**0,271**	**0,221**	**0,251**	**NA**	**0,27**	**0,258**	**0,273**		**0,04**	**0,031**	**NA**
	0,079	0,093	0,099	0,112	0,09	0,112	0,078	0,077	0,073	0,069	0,088	0,069	0,089	0,056		0,023	0,018	0,019
ASIA-3	**0,384**	**0,408**	**0,388**	**0,071**	**0,328**	**0,254**	**0,343**	**0,321**	**0,336**	**0,352**	**NA**	**0,328**	**0,323**	**0,353**	**0,321**		**0,037**	**NA**
	0,102	0,113	0,124	0,045	0,054	0,025	0,099	0,091	0,084	0,112	0,108	0,09	0,106	0,103	0,109		0,02	0,022
RCK-L	**0,194**	**0,205**	**0,175**	**0,246**	**0,138**	**0,134**	**0,104**	**0,149**	**0,116**	**0,223**	**NA**	**0,205**	**0,152**	**0,164**	**0,159**	**0,246**		**NA**
	0,03	0,046	0,052	0,093	0,036	0,087	0,028	0,028	0,03	0,067	0,045	0,046	0,046	0,032	0,061	0,084		0,013
ASIA-4	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	
	0,056	0,067	0,069	0,108	0,054	0,106	0,054	0,05	0,046	0,093	0,071	0,072	0,072	0,048	0,081	0,102	0,131	

NA-4: North America-4; SA-4: South America-4; SA-3: South America-3;VAC: Vaccine; SA-2: South America-2; NA-1:North America-1, NA-2:North America-2;NA-3: North America-3; EU/SA-1:Europe/South America-1; ARC-L:Arctic-Like; EU/WL: European Wildlife; AFR-1: Africa-1; AFR-2: Africa-2;RCK-L: Rockborn-Like. In bold are the values of the distances of Fsp, below are the values of the distances of the H gene. Blue color shows the estimated standard error, was established with a Bootstrap of 1000 replicas. NA: values not available due to lack of sequences for Fsp.

In this study, three patients had incomplete vaccination, two sequences were grouped in the “South America/North America-4” lineage, of which one strain has a very extensive branch (Figure 2 - Sequence Mde-19a-CO-2017), another one is related to CDV strains circulating in North America (Figure 3- Sequence Mde-18a-CO-2017) and the last one is 1aM-CO-2017 belonging to the South America-3 lineage (Figure 3). A comparison between the vaccine and these Colombian strains in the linear hemagglutinin noose epitope (HNE) showed the presence of multiple substitutions in this epitope (Figure 4). Sequence 18a-CO-2017 presents the following substitutions: A367V, E372D, G376N and T386S, while strain 1aM-CO-2017 of the South America-3 lineage presents this substitution A367V, G376N, T386S.

Figure 4. Alignment of the hemagglutinating region of the CDV protein H. Alignment of the deduced amino acid sequence located between residues 364 and 392 of the CDV hemagglutinin protein between the vaccine strains and the North / South America-4 and South America-3 lineages.

3.5 Amino acid analysis of the Fsp

The Fsp peptide possesses 95 variable aminoacids (from 135 total). We found 16 substitutions that are exclusive to the South America-3 lineage: S9P, T32I, A35S, T40P, D54N, R55K, S58N, Y59H, M61T, R67F H80C, H83R, I102S, Q115H, C116F, L129F. For the South America/North America-4 lineage, 20 exclusive substitutions were found: D28S, E29G, A35T, N62D, S71G, H80R, V94I, N108T, S112P, S114P, K134E. Moreover, a different set of aminoacid subtitutions were found with an specific geographic pattern only in South America-4: T13V, T40K, S45F, S58Q, V79I, S95P, R105W, G113C. Besides, in

the most identical strains of North and South America-4 we only found the substitutions T13M and N76S, thus showing autopomorphic characters.

3.6 CDV H and Fsp glycosylation analysis

Potential glycosylation sites for the CDV H protein from South America-3 lineage has been previously reported. No new potential glycosylation sites were found in the 2017 samples belonging to this lineage in new sampling areas of Colombia. For the North America/South America-4 lineage we found the presence of eight potential glycosylation sites (NXS/T) at positions 19-21, 149-151, 309-311, 391-393, 422-424, 456-458, 587-589, 603-605 which are common to other lineages. The previously reported Asia-1 potential glycosylation sites at position 584-586 (Mochizuki et al., 1999) was not present in the sequences of the North American/South America-4 lineage.

On the potential glycosylation analysis of the Fsp peptide from South America-3 lineage, we found the presence of two potential glycosylation sites (NXS/T) at positions 62-64 and 108-110 that are common to the other lineages. However, none potential glycosylation sites were found in the any of the South America/North America-4 sequences.

3.7 CDV H and Fsp Sites under positive selection

We evaluated the non-neutral selection by the FUBAR method. For the H gene, we found that sequences displayed three sites under positive selection: 522, 549 and 582 with a posterior probability of 0.9 and a Bayes factor of 28.3, 220.9 and 44.7 respectively. We also found 247 sites under negative selection with a posterior probability of 0.9 and Bayes Factor <1.

For the Fsp coding region, we found nine sites under positive selection under FUBAR method: 21,39,46,51,79,98,99,101,102 with a posterior probability of 0.9 and a Bayes factor of 67, 306 and 88 in sites 21, 76 y 98 respectively (Figure 5). Likewise, we found that Fsp coding region has five sites under negative selection: 45, 83, 89, 121, 133 with a posterior probability of 0.9 and Bayes Factor <1.

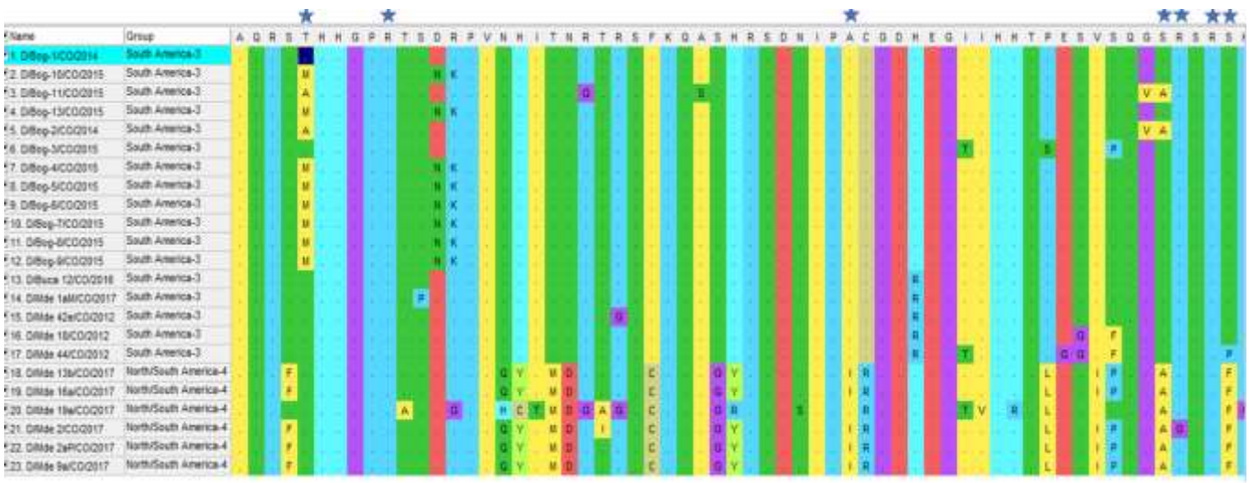

Figure 5. Alineamiento de la region Fsp de CDV. Alignment of the deduced amino acid sequence located between residues 42 and 102 of the CDV Fusion protein between the North / South America-4 and South America-3 lineages. The Upper Star indicates the sites under positive selection.

3.8 H gene and Fsp phylogeography

For this analysis, the vaccine sequences were eliminated since there were no exact dates of isolation of these strains and this produces biases in the substitution rates. Table 4 shows the evolutionary model for each gene. Also, table 5 shows the estimated tMRCA for the H gene. The South America-3 and South/North America-4 lineages, have a tMRCA corresponding to 1964 and 1925, respectively (see table 5). Furthermore, the phylogeography of the Fsp and H gene, respectively, is shown in figures 6 and 7.

Table 4. Evolutionary Parameters genes H and Fsp

GEN	Evolution model	Evolution rate		Rate dN/dS	tMRCA	HDP 95% Interval
		Mean	HDP 95% Interval			
H	T92+G	4,87 X 10^{-4}	3,78 X 10^{-4} - 5,94X 10^{-4}	0,024	1900	1873-1925
FSP	HKY+G	1,642 x 10^{-3}	1,12 X 10^{-3} - 2,12 X 10^{-3}	0,4	1909	1872-1942

Table 5. H gene molecular clocks

Lineage	tMRCA		# Sequences
	Mean	HDP 95% Interval	
North America-1	1998	1996-1997	2

North America-2	1957	1947-1965	3
North America-3	2005	n/a	1
Asia-1	1971	1961-1980	6
Asia-2	1991	1986- 1996	5
Asia-3	1999	1994- 2003	2
Asia-4	1979	1969- 1988	3
Africa-1	1982	1973- 1991	4
Africa-2	1985	1979-1990	3
Arctic -Like	1992	1986-1997	4
European wildlife	1975	1965- 1985	2
Rockborn-Like	1944	1932-1956	2
Europe/South America-1	1975	1966-1982	6
South America-2	2005	n/a	1
South America-3	1964	1943-1983	8
South/North America-4	1925	1891-1955	7

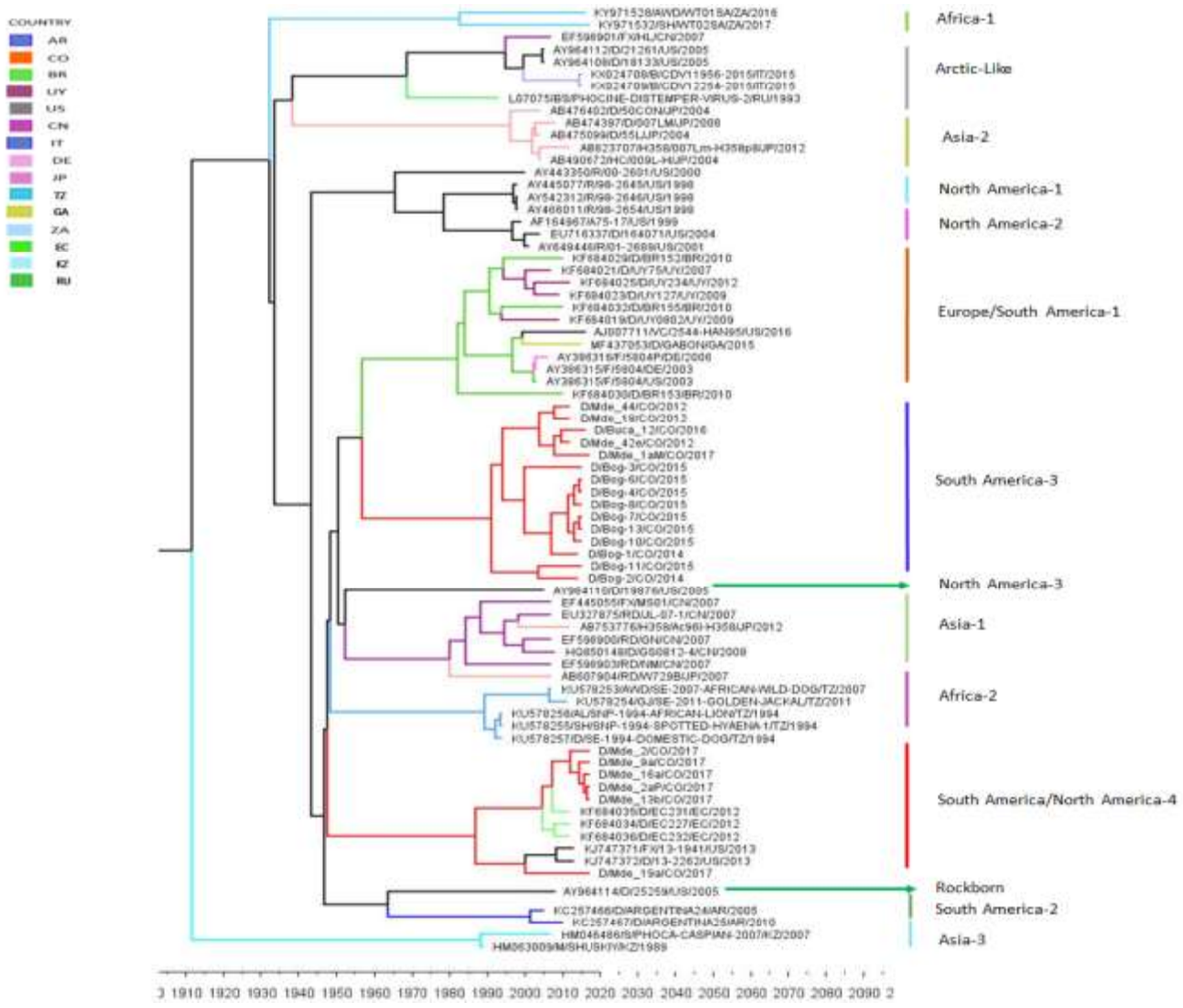

Figure 6. Time-measured Bayesian MCC tree for CDV Fsp Fragment. The branches are colored according to the country color code in the upper left position. The Colombian sequences are depicted in red.

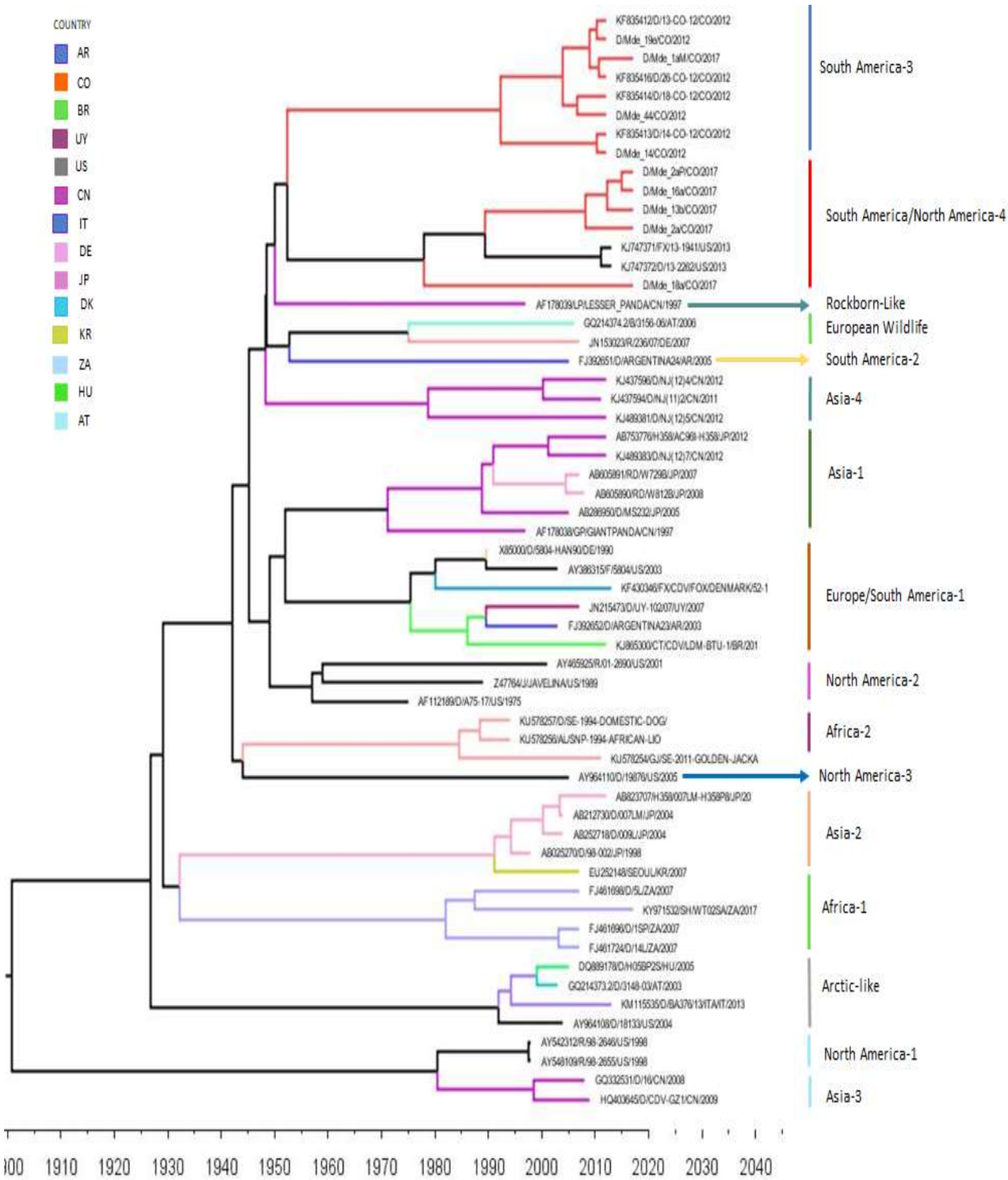

Figure 7. Time-measured bayesian MCC tree for CDV H gene. The branches are colored according to the country color code in the upper left position. The Colombian sequences are depicted in red.

Phylogenetic characterization of CDV is carried out based on the H gene sequence because

4. Discussion

it presents high nucleotide variability in comparison to other Paramyxoviruses and between the field strains and the vaccine strains (Bolt et al. 1997; Pomeroy et al. 2008). By this, it is accepted that two strains belong to the same lineage when their amino acid diversity is less than 4% (Bolt et al. 1997), obtaining a classification of worldwide circulating strains in 17 lineages with a geographical distribution pattern (Martella et al. 2006; Nikolin et al. 2016; Riley and Wilkes 2015; Espinal, Díaz, and Ruiz-Saenz 2014; Anis, Newell, et al. 2018). However, a fragment that codes for the Fusion Protein Signal-Peptide-Coding Region (Fsp) of the CDV, has been reported as an alternative for the classification of the CDV strains since it is highly divergent, and has shown a classification similar to that performed with the H gene. By using this approach, it has been reported that two strains belong to the same CDV lineage if the amino acid divergence is less than 19% (Sarute et al. 2013).

In 2012, based on samples from Medellín city, (Espinal, Díaz, and Ruiz-Saenz 2014) characterized the South America-3 lineage using the full H gene sequencing approach; in that study it was not possible to compare the circulation of this CDV lineage in other regions of Colombia or in closer countries such Ecuador. In the present study, the Fsp coding region was sequenced from samples taken in Medellín between 2012 and 2017, and from other Colombian cities such as Bucaramanga (2016) and Bogotá (2015); stablishing the wide circulation of the CDV South American Lineage 3 in these regions (Figure 2). Agreeing with (Sarute et al. 2013), The phylogenetic analysis, showed the accepted lineage classification when presenting an amino acid divergence in the strains between 1.5-6.7% (data not shown).

Analyzing the phylogenetic tree of the Fsp coding region from samples taken in Medellín in 2017, it was observed that sequences showed a monophyletic group with the strains of Ecuador and the America-4 lineage, evidencing a 12% amino acid divergence supported in the table of distances (Table 3), which lead us to the conclusion that those viral sequences belong to the same lineage. However, to give greater support to this finding, were amplified the H gene in the same 2017 samples. In the phylogenetic tree of H gene, we observed a

monophyletic group together with the strains of the Lineage America-4, presenting an amino acid divergence in the H gene of 2.8%, showing that these strains belong to the same CDV lineage, for this reason we suggest to call this lineage “South America/North America-4”.

Comparing the topologies of the phylogenetic trees, we used as outgroup the North America-1/Vaccine/Asia-3 Lineage. We observed that although the lineages characterized in this work are sister groups, they present differences of ancestor, so in the H gene tree, the oldest clade are the Africa-1 lineages and the Arctic-like and Asia-2 groups. From this node, the Asia-4 lineage and the South America-3 and “South America/North America-4” lineages appear as sister groups. A polytomy emerges that originated the lineages North America-3, South America-2 as sister groups, North America-2, wild Europe, Asia-1 and Rockborn-Like as sister groups and Africa-2 and Europe/South America-1 as sister groups.

In the Fsp coding region tree, the ancestral clades of the characterized lineages arise from a polytomy that gives originates the Arctic-Like and Asia-2 lineages as sister groups, Africa-1 and the third node that gives origin to the lineages North America -1 and North America-2 as sisters groups and the Rockborn-Like lineage (Figure 2), and from this node another polytomy is presented giving rise to the lineages Europe/South America-1, South America-2, Africa-2, and as sister groups Asia -1 and North America-3, and the lineages South America-3 and “South America/North America-4” as sister groups. In 2013, Sarute *et al.* describe topological differences between the Fsp and H gene trees (Sarute et al. 2013), although topological structures do not correspond completely to those found in our study (Figures 2 and 3), it should be taken into account that nowadays, there are a higher number of Fsp coding sequences representing most of lineages characterized to date.

By using the CDV classification of "subgenotypes" as in Measles, we can define that a subgenotype is conformed by sequences that have an amino acid identity of the H gene of 98% and a high value of Bootstrap> 70% (Budaszewski et al. 2014). Within these criteria, we can point out that in both Lineages South America-3 and “North America/South America-

4", presents three subgenotypes. Otherwise when we arbitrarily extrapolate this classification to the Fsp region, a different set of subgenotypes where found (A to E for South America-3 and A to C in the "North America/South America-4" lineage). Those results show lower number of Subgenotypes in comparison to the Europe/South America-I lineage for which has been reported the presence of at least eight (A to H) subgenotypes, including CDV H gene sequences from at least seven different countries (Budaszewski et al. 2014).

In Measles, different subgenotypes are not geographically restricted although some appear to be the mainly 'endemic' in different areas of the world (B. K. Rima et al. 1995). In the present study, it was not possible to establish a geographic pattern in the classification between subgenotypes based on the H gene as has been suggested for the Europe/South America-1 and Southern Africa subgenotypes (Oliferenko et al. 1999; Loots et al. 2018) and for Measles (Riddell, Rota, and Rota 2005). However, with the Fsp fragment, this distribution can be observed between regions (Figure 2). Subgenotype 3A circulating only in Medellín, subgenotype 3D circulating only in Bogotá. Also, in the lineage "North America/South America-4", the subgenotype 4A was only reported in the Colombian strains and the subgenotype 4D in Ecuadorian strains. A higher number of CDV sequences collected from different areas of those countries are necessary for a better understanding about the circulation history of CDV subgenotypes in the Americas.

A temporary pattern of distribution of Measles virus genotypes and subgenotypes has been reported (B. K. Rima et al. 1995; Riddell, Rota, and Rota 2005; Dia et al. 2015). also, for CDV temporary distribution patter has been reported for some of the Europe/South America-I and Southern Africa subgenotypes (Budaszewski et al. 2014; Loots et al. 2018). Our results showed a temporary pattern of distribution in most of the subgenotypes as for South America-3 as for the "South America/North America 4" lineage (Figures 2 and 3). Those results must be evaluated carefully due that although it could be representing a temporary patter of CDV distribution and a possible strain displacement pattern, sampling bias could be a possible explanation for this scenario in our samples. International routine determination of CDV Lineages and subgenotypes plus molecular surveillance can be useful to propose a more accurate epidemiologically understanding of CDV temporal distribution.

The uncontrolled commercialization of puppies from South America to the USA could be the route of transmission of the "South America/North America-4" lineage in these two regions of the continent. It is important to highlight that this is the second reported lineage that are actively circulating in two different continental regions, like the Europe/South America-1 lineage (Panzera et al. 2012). It would be very important to establish wider phylogeography studies of the "South America/ North America -4" lineage to establish the origin and geographical spread throughout the American continent in order to know if this CDV lineage rise Ecuador and ascended through Colombia to the USA or if these viruses descended to South America from the USA. Since the CDV is a re-emerging infection in the USA, with at least five different lineages circulating (Anis, Newell, et al. 2018), deeper phylogenetic analysis could help to understand the epidemiology of CDV in the continent.

As we previously reported (Espinal, Díaz, and Ruiz-Saenz 2014), BLAST analysis of H sequence data from Colombian commercial vaccines used as positive controls in this study revealed that vaccines had 99 -100% identity with the vaccines strains form North America-1 Lineage and one vaccine showed a 99% identity with a Rockborn strain.

In this study, an amino acid divergence close to 11% in the H protein was obtained between vaccine strains and South America-3 and "South America/North America -4" lineages (Table 3). Although CDV is currently recognized as a single serotype (Timm C. Harder and Osterhaus 1997) and there is not enough evidence of antigenic divergence as a result of genetic divergence. It was recently reported significant differences in the evaluation of neutralizing titers comparing "South America/North America -4" lineage strains to one of the America 1 type vaccine (Anis, Holford, et al. 2018). Taking into account those results and the fact that multiple recognized CDV cases has been recorded in vaccinated animals (Kapil et al. 2008; Gámiz et al. 2011; Budaszewski et al. 2014; Espinal, Díaz, and Ruiz-Saenz 2014; Riley and Wilkes 2015), it is necessary to perform wider and updated antigenic analysis of CDV to understand the antigenic differences between the multiple worldwide circulating lineages and a possible vaccine update that included most prevalent antigenic types.

In the positive selection analysis, we observed that the "South America/North America-4" lineage possesses a unique substitution (V79I) in the Fsp fragment, site that is under positive selection; Also, the South America-3 lineage also possesses a unique substitution I102S,

which is under positive selection, determined with the FUBAR method, which assumes that the selection pressure for each site is constant throughout the phylogeny (Murrell et al. 2013). Likewise, it was determined that the sites 98,99,101,102 of Fsp (Figure 5) are under positive selection, this epitope must be studied to understand the role of this changes in vaccine failures and interspecies host changes.

The linear hemagglutinin noose epitope (HNE) located at amino acid 364-392 of CDV H protein of is a conserved epitope in the morbilliviruses (Santibanez et al. 2005). This is the region of the H protein that is recognized by antibodies, (Sugiyama et al. 2002; Veronika Von Messling et al. 2005; Tahara et al. 2013). Recently, it has been suggested that substitutions in this epitope may interfere with the ability of the vaccine to provide adequate protection against infection with wild type viruses (Anis, Newell, et al. 2018). As reported recently for the "South America/North America-4" strains, we observed the presence of multiple substitutions in the HNE epitope of CDV viruses infecting vaccinated animals (Figure 4). However, from the bioinformatic approach used in this study, we can only propose that these substitutions could be interfering with the neutralization capacity of the vaccine against wild strains, for this reason it is necessary to perform Neutralization studies in this region of vaccines with wild strains. Currently, structural biology studies are underway in Colombian CDV viruses to understand the role of structural substitutions in the HNE epitope of Colombian CDV viruses and its role in viral neutralization.

CDV has one of the highest reported substitution rates in the *Paramixoviridae* Family ($10.53–11.65 \times 10^{-4}$ Substitutions/Base/Year) (Pomeroy et al. 2008). Our results show that CDV in Colombia possess a high variability including the circulation of two lineages and multiples subgenotypes (Figure 2-3 and Table 3). Our sampling strategy does not have enough distribution in time and/or geographic distribution in order to accurately explain the variability of CDV in the region. However, considering that the H gene has undergone genetic drift in different geographical regions (Martella et al. 2006) we hypothesize that selective and nonselective processes can play important roles in the co-circulation of multiple lineages in an area as has been reported (Ke et al. 2015).

Phylogeographic analysis for the Fsp region and the H gene shows an evolutionary rate for the H gene similar to that reported perviously by (Panzera et al., 2015). However, the two trees of the phylogeography differ in the topology whereby the ancestry of the lineages of interest is very different (Figures 6 and 7). These differences in topology in comparison to others (Fischer et al., 2016) may be due to the fact that in the present analysis we exclude Vaccine strains reported in recent dates, and we added three newly reported lineages including South America / North America-4 in that phylogeography analysis. In describing the circulation of the South America/North America-4 lineage by comparing both trees we observed an apparently first circulation in Colombia and Ecuador, then a circulation of this viral lineage in the United States and again in Colombia (Figures 6 and 7). It is unclear if the variation in spatiotemporal sampling of the South America/North America-4 lineage more likely to bias as has been previously reported in other viral models (Magee, Taylor, and Scotch 2018). In addition, since we suspected that the most ancestral Colombian sequences of both trees are an immune escape mutant, deeper analyses must be performed avoiding misleading results regarding the dynamics of the CDV South America/North America-4.

Outbreaks of CDV occurs in endemic and acute epidemic cycles leading transmission in susceptible host populations. In the presence of full or partial vaccination lifelong immunity could lead to the survival of the remaining coexisting lineages driven by nonselective epidemiological processes (B. K. Rima et al. 1995). Our results showing no and/or incomplete vaccination supports this hypothesis (Table 2).

In contrast with measles, the only natural host of which is humans, broad ranges of host species are susceptible to CDV infection, which results in complications in terms of selection pressure for this virus. It is noteworthy that CDV “South America/ North America -4” lineage characterized in the USA were obtained from domestic dogs and foxes, indicating that this lineage have the ability to jump the Species Barrier (Riley and Wilkes 2015; Martinez-Gutierrez and Ruiz-Saenz 2016). In Colombia, there are reports of CDV infection in wildlife (Varela Arias et al. 2015), however, no phylogenetic analysis has been performed in viruses belonging to those infected animals.

The reported substitutions in circulating CDV protein H in wildlife include the sites E276V, Q392R, R519I, I542F, Y549H, sites that possess the same amino acids in the South America-3 and South America/NorthAmerica-4 lineages, which indicates the potential of jump of the species barrier in these two lineages, however there is no statistical association that demonstrates these hypotheses (Nikolin, Wibbelt, et al. 2012).

In conclusion, we are reporting the co-circulation of two CDV lineages in Colombia: Lineage South America-3 circulating in Medellín, Bucaramanga and Bogotá, and the concurrent circulation of a new lineage not described in the country that is mainly infecting dogs in Medellín. This last lineage is evolutionarily related to strains reported in domestic dogs in Ecuador and in domestic dogs and wildlife in the USA. Taking the intercontinental circulation of this lineage we propose to denote this “South America/North America-4”

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Authors ' contributions

JRS and PB conceived the study. JDV NRF, FJD, EM, PB and JRS were involved in all other aspects of the study, including data collection, data analysis, drafting and editing the paper. All authors read and approved the final manuscript.

Conflict of interest statement: The authors declare that they have nothing to disclose

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6.CAPITULO III

En este capítulo se presenta el trabajo desarrollado para dar cumplimiento del segundo, tercer, cuarto y quinto objetivos específicos de la tesis:

2.Describir la secuencia de los genes F, P y N del CDV Linaje Suramérica 3.

3.Explorar las relaciones filogenéticas del CDV Linaje Suramérica 3 en secuencia extendida.

4.Explorar la presencia de mutaciones del CDV Linaje Suramérica 3 asociadas a la adaptación a células humanas, salto de la barrera de especies y patogenicidad.

5. Describir la presencia de sitios de recombinación en la secuencia extendida del CDV Linaje Suramérica 3.

Análisis filogenómico del virus del distemper canino en Colombia: Evidencia de recombinación entre dos linajes co-circulantes

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Resumen

El CDV es una enfermedad considerada re-emergente en caninos domésticos al presentar alta divergencia entre las cepas circulantes a nivel mundial. Así en Colombia co-circulan los linajes Suramérica-3 y Suramérica/Norteamérica-4 en caninos domésticos, ambos caracterizados en el Área metropolitana de Medellín, dando la posibilidad de presentarse co-infección en los pacientes. En este trabajo se secuenciaron dos genomas de CDV de cepas circulantes en Medellín por el método de secuenciación Sanger, exploramos la relación filogenética con las secuencias de genomas disponibles, describimos la presencia de mutaciones del CDV Linajes Suramérica-3 y Suramérica/Norteamérica-4 asociadas a la adaptación a células humanas, salto de la barrera de especies y patogenicidad, además establecimos las tasas evolutivas y tiempo del ancestro común más cercano para cada gen y encontramos evidencia de recombinación homóloga entre dos linajes diferentes de CDV co-circulantes y caracterizamos múltiples sitios bajos selección positiva.

Palabras claves: filogenómica, co-infección, re-emergente, recombinación homóloga

.

Introducción

El CDV pertenece a la familia *Paramyxoviridae*, género *Morbillivirus*, en el cual se incluyen otros virus como el sarampión, el distemper de las focas, peste bovina y virus de los pequeños rumiantes, que son de importancia epidemiológica para poblaciones humanas y animales (MacLachlan and Dubovi 2011). El CDV es un virus RNA de cadena sencilla en sentido negativo, el genoma del CDV está formado por 15690 nucleótidos que incluyen 6 genes que codifican para las proteínas estructurales : la nucleocápside -N- (encapsula el RNA viral), la fosfoproteína -P- (cofactor proteico de la polimerasa viral), la proteína de matriz -M- (capa interna de envoltura viral), proteína de fusión-F-(fusión entre la envoltura viral y la membrana del hospedero), la Hemaglutinina-H- (tropismo celular) y la proteína grande -L- (polimerasa viral) (MacLachlan and Dubovi 2011). Además, el gen P codifica dos proteínas no estructurales: proteína C y V, que inhiben la respuesta de los interferones tipo I y II (Schuhmann, Pfaller, and Conzelmann 2011; Chinnakannan, Nanda, and Baron 2013; Yokota, Okabayashi, and Fujii 2011).

Para la clasificación filogenética del CDV se ha utilizado el gen H, el cual evidencia una gran diversidad genética con un patrón de distribución geográfico (Martella, Elia, and Buonavoglia 2010; Espinal, Díaz, and Ruiz-Saenz 2014), así a las fecha se han descrito 17 linajes del CDV a nivel mundial: Norteamérica 1-5, Europa/Suramérica 1, Suramérica 2 y 3, Europa silvestre, Tipo Artico, Tipo Rockborn, África 1 y 2, Asia1-4 (Espinal, Díaz, and Ruiz-Saenz 2014; Pope et al. 2016; Nikolin et al. 2016; Ke et al. 2015; Bolt et al. 1997; Martella et al. 2006; Anis, Newell, et al. 2018).

Debido a la creciente necesidad de conocer el impacto de la evolución viral y el salto en la barrera de especies sobre los diferentes genes del CDV, el estudio de genomas completos mediante filogenómica ha permitido inferir las relaciones existentes entre las cepas, la historia evolutiva y dilucidar algunos mecanismos de evolución molecular (substitución, inserción, deleción, recombinación, duplicación, inversión, transposición y translocación) involucrados en la evolución de Virus RNA (Philippe et al. 2005; Delsuc, Brinkmann, and Philippe 2005). Adicionalmente, se ha ampliado el conocimiento de características y substituciones genómicas implicadas en el paso entre diferentes hospederos y entre fauna doméstica y fauna silvestre (Nikolin et al. 2016) . Aunque la disponibilidad de secuencias de genoma completo del CDV aún es reducida (83 genomas completos reportados en el GenBank) en comparación con la disponibilidad de secuencias del gen H, encontramos que con excepción de los linajes tipo Rockborn, Norteamérica-3 y 5, Fauna silvestre europea, Suramérica-2 y Asia-4 la mayoría de los linajes descritos a nivel mundial se encuentran bien representados y correctamente reportados (Picket et al. 2012). En el presente estudio caracterizamos el genoma completo de dos linajes del CDV que se encuentran circulando en Medellín, Colombia.

2. Materiales y métodos

2.1. Tipo de estudio y Consideraciones éticas

Este estudio es descriptivo y retrospectivo. Adicionalmente este estudio fue aprobado por el Comité de Ética para la Experimentación Animal de la Universidad Cooperativa de Colombia en Bucaramanga. Los propietarios de los perros firmaron formularios de consentimiento informado aprobados por el comité de ética. Además, los autores declaran que la implementación de este trabajo siguió todas las reglas científicas, técnicas y administrativas para la investigación con animales.

2.2 Especímenes clínicos

De un muestreo realizado en el Área Metropolitana del Valle de Aburrá, Antioquia en el año 2012 se obtuvo la Cepa MDE-44/12 la cual pertenece al Linaje South America-3 (Espinal, Díaz, and Ruiz-Saenz 2014) . De un segundo muestreo realizado en el Año 2017, se obtuvo la cepa MDE-02/17, la cual pertenece al Linaje Sur América/Norte América-4.

2.3 Extracción de ARN

El ARN total se extrajo de 140 µl de sobrenadante de secreción nasal y ocular mediante el procedimiento Mini Spin de QIAamp Viral RNA (QIAGEN®, Hilden, Alemania) de acuerdo con las instrucciones del fabricante. La calidad y cantidad del ARN se determinó mediante análisis espectrofotométrico con un espectrofotómetro NanoDrop ™ One UV-Vis (Thermo Scientific, Wilmington, Delaware, EE. UU.) Y se almacenaron alícuotas de ARN a -80ºC hasta su uso.

2.4 Síntesis de ADN complementario (ADNc)

La síntesis de ADNc se realizó utilizando el kit de síntesis de cDNA RevertAid ™ Premium (Thermo Scientific®, Glen Burnie, MD) de acuerdo con las instrucciones del fabricante. Brevemente, una mezcla de desnaturalización que consiste en 1 µl (100 pmol / µl) de hexámeros aleatorios, 1 µl de mezcla dNTP (10 mM) y 13 µl (0.02-4.6 µg) de ARN total se desnaturalizó inicialmente a 65 ° C durante 5 min. e inmediatamente se incuba en hielo. La solución de mezcla RT consistió en 4 µl de Tampón de Transcriptasa Reversa 5X y 1 µl de Mezcla de Enzima Premium RevertAid ™. La mezcla RT se añadió a la mezcla de desnaturalización y la transcripción inversa se realizó en un volumen total de 20 µl en un termociclador de PCR ProFlex ™ (Applied Biosystems®, Foster city, California, EE. UU.) Durante 10 min a 25 ° C seguido de 30 min a 50ºC; la reacción se terminó calentando a 85 ° C durante 5 min. El producto de reacción se almacenó a -80ºC hasta su uso.

2.5 PCR y secuenciación

Para amplificar genoma completo se usaron un conjunto de 15 pares de cebadores reportados previamente por (Riley and Wilkes 2015) los cuales presentan 50 pb superpuestas, amplificando un segmento de 1000 pb aproximadamente (menos los extremos no codificantes 5 'y 3') ver tabla 1.

Tabla 1. Conjunto de primers usados para amplificar genoma de CDV

SET	FORWARD	REVERSE	POSICIÓN GENÓMICA *
F1/R1	GAGAACAAGGTC AGGGTTCAG	TTGCCGGCAGATC TTCTAAC	77–1231
F2/R2	TGCTATGGGAGT TGGTGTTG	TCTTCGCCAGAAT CCTCAGT	1112–2273
F3/R3	CGAAGATGCTGA CAGTCTCG	GAAAGCAGTTCTG TGCCTGTT	2169–3368
F4/R4	CAAAGTCACAAA CACAACATGC	GGGACTGATGGTT GCAAGAC	3223–4372
F5/R5	TGTTACCCGCTC ATGGAGAT	CCAAGTACTGGTG ACTGGGTCT	4272–5433
F6/R6	GGTGCATTGGAA TAGCCAGT	GCAGGTATCGGAG GCAATAA	5299–6530

F7/R7	CTTGGTGTCTGG GACGATG	GCTGCCGATGCAA TAGATTT	6377–7578
F8/R8	GGTTACGGTTGC CACAAAAA	TCCATAATCTGGG ATGTTTGAA	7389–8580
F9/R9	AAGAACGGAACA GTCCTTGG	TTGTGACTGGTGA GGTCAATG	8441–9685
F10/R10	TGCAAAGCTCAC AGTGGTTC	CAGGCTCGCATTT TGTAGGT	9496–10696
F11/R11	GAGGACTCTCAG TTTGACCCTTA	CACATCCCGTGTC ATAGCTG	10539–11777
F12/R12	CCGCATGCAGTA ACATTTCA	GCCCATGAGTACA CAGTTGCT	11626–12772
F13/R13	TACATCGGGTCC ACAACAGA	CCTCATCACTTTC GCACAAA	12663–13821
F14/R14	CACGGACCCTCT CTTGACTC	CGAGGTAGGCCTC TGTTGAC	13683–14901
F15/R15	GCGACTGGGTTC AAGGATTTA	AGCAATGAATAGC AGAGGGTTAG	14797–15623

***Genoma de referencia: AF164967 (A75/17)**

Se añadieron 4 µl de ADNc a una mezcla de reacción de PCR que consistía en 25 µl de mezcla PCR Maxima Hot Start (2X), 15 µl de agua sin nucleasas y 3 µl (10 µM) de cada uno de los cebadores (Tabla 1). La PCR se realizó en un termociclador de PCR ProFlex ™ (Applied Biosystems®) bajo las siguientes condiciones: desnaturalización inicial a 95ºC durante 4 minutos, seguido de 35 ciclos de desnaturalización a 95ºC durante 30 segundos, alineamiento a 60°C durante 60 segundos, extensión a 72ºC durante 3 min, y una extensión final a 72ºC durante 10 min.

Después de la PCR, se realizó la electroforesis en la cual se adicionaron 5 µl de los amplicones en un gel de agarosa al 1,5% (AGAROSE I ™, Amresco, Solon, OH, EE. UU.) y fueron corridos a 110 V durante 60 min. Los geles se tiñeron usando el iuntercalente de DNA EZ-VISION™ (Amresco® Solom, OH, EE. UU.) Y se observaron mediante transiluminación con luz UV utilizando el sistema GelDoc TM XR + con el software de adquisición de imágenes ImageLab ™ (Bio-Rad, Hercules, CA, EE. UU.). Los tamaños del producto de amplificación se estimaron usando una escala de peso molecular de 100 a 3000 pb (GeneRuler ™ 100 pb Plus DNA Ladder, Thermo Scientific®).

Los amplicones de la PCR se enviaron para su purificación y secuenciación a Macrogen Inc., (Macrogen Inc., Seúl, Corea) se usaron los mismos primers de la PCR para la secuenciación y se usó un secuenciador automático ABI3711 ™ (Macrogen ™).

2.6 Análisis filogenético

Los datos obtenidos de la secuenciación fueron ensamblados y editados usando el programa SeqMan (paquete de software DNAStar Lasergene ™ V15.0, Madison, Wisconsin, EE. UU.). Se usó Nucleotide BLAST (herramienta de búsqueda de alineación local básica) para explorar la similitud de secuencia de cepas de CDV colombianas con todas las secuencias disponibles de CDV en las bases de datos de nucleótidos de NCBI. El alineamiento de 15690 nucleótidos y sus aminoácidos deducidos para las 2 secuencias de genoma de CDV de cepas Colombia, junto con los 83 genomas disponibles a nivel mundial, se realizó con el programa MEGA 7 (Kumar, Stecher, and Tamura 2016), se usó el algoritmo ClustalW y se calcularon las distancias no corregidas (p) de nucleótidos y aminoácidos .

Para el análisis filogenético para cada gen y genoma de CDV, se corrieron siete modelos evolutivos (genoma CDV = GTR + G + I, L= GTR+G, H=T92+G, F=T92+G, , M= T92+G, P=TN93+G, N= T92+G) en el programa MEGA 7 (Kumar, Stecher, and Tamura 2016). Para realizar los árboles filogenéticos se usaron métodos de distancias Neighborn Joining (NJ) y de caracteres Máxima verosimilitud (ML) y se confirmaron por el método Mr. Bayes. En todos los métodos se usó Bootstrap de 1000. Las secuencias de Linaje Norte America-1 se usaron como grupo externo en los siete modelos.

2.7 Análisis de recombinación

Los eventos de recombinación se evaluaron usando RDP4.0 (Martin et al. 2015) y se confirmaron mediante los subprogramas de Simplot v3.5.1 (Lole and Bollinger 1999). Posteriormente, los árboles ML fueron construidos en el paquete MEGA 7.0 (Kumar, Stecher, and Tamura 2016).

2.8 Análisis de aminoácidos del CDV

Las secuencias de aminoácidos deducidas a partir del genoma de las cepas colombianas de CDV de tipo silvestre (5230 aa) se alinearon con múltiples secuencias de genomas CDV de diferentes regiones geográficas utilizando MEGA 7. Se evaluó el perfil de aminoácidos y las diferencias potenciales con las cepas vacunales y las cepas silvestres de los linajes de CDV ya conocidos. Adicionalmente, se evaluó la presencia de mutaciones del CDV en linajes Colombianos asociadas a la adaptación a células humanas, salto de la barrera de especies y patogenicidad. La predicción de posibles sitios de glicosilación ligada a N se realizó con el paquete NetNGlyc 1.0 (Gupta, Jung, and Brunak 2017).

2.9 Análisis de presión de selección

Para identificar los sitios aminoacídicos en las proteínas de CDV con selección positiva se calculó la relación de las sustituciones no sinónimas (dN) a las sinónimas (dS) utilizando la reconstrucción filogenética ML y el modelo de sustitución de nucleótidos reversible general disponible a través de programa web Datamonkey (Murrell et al. 2013). Para detectar la selección no neutral, la aproximación bayesiana no restringida rápida (FUBAR), se implementó dentro del paquete de software HyPhy del programa Datamonkey. El rango de significancia de la probabilidad posterior está entre 0-1. En general, las probabilidades posteriores > 0,9 sugieren fuertemente la selección positiva y el factor de Bayes = 50 se utilizó para estimar las tasas de dN y dS dentro de cada codón. Los valores dN / dS> 1, dN / dS = 1 y dN / dS <1 se utilizaron para definir la selección positiva (evolución molecular

adaptativa), mutaciones neutrales y selección negativa (selección purificadora), respectivamente.

2.10 Relojes moleculares

Se determinó para cada gen la tasa de sustitución media (sustituciones por sitio por año) y el tiempo al ancestro común más reciente (tMRCA), de los linajes de CDV usando un enfoque Bayesiano de la Cadena Markoviana Monte-Carlo implementado en el paquete BEAUti / BEAST v1.8.4.(Drummond et al. 2012). El análisis fue implementado usando un reloj molecular estricto con un tamaño de población constante, se corrieron 3 $x10^7$ generaciones con el fin de asegurar el tamaño efectivo de la población (ESS) mayor a 200 para los parámetros evaluados usando el programa Tracer v1.7 (Rambaut et al. 2018).

3.Resultados

3.1 Especímenes clínicos

Se amplificaron dos genomas obtenidos de muestras clínicas de secreción ocular y nasal de muestreos realizados en 2012 y 2017 de la ciudad de Medellín, respectivamente. El paciente de 2012 tuvo sintomatología respiratoria y neurológica; el paciente de 2017 presentaba sintomatología respiratoria y digestiva; en ambos pacientes se desconoce su esquema de vacunación.

3.2 Análisis filogenético de genoma completo

Se logró secuenciar 15525 nucleótidos del genoma de CDV en las dos muestras clínicas, las cuales fueron comparadas con 56 genomas de los 83 disponibles en GenBank, ya que al realizar el árbol filogenético preliminar se descartaron genomas similares pertenecientes a diferentes linajes distribuidos mundialmente.

Los análisis filogenéticos lograron identificar 11 de los 17 linajes descritos mundialmente, dado que no se encuentran reportadas secuencias de genoma completo de los linajes Suramérica-2, Norteamérica-3 y 5, Fauna silvestre europea, Tipo Rockborn y Asia-4 (Figura 1). Al analizar la topología del árbol tenemos como grupo externo las cepas vacunales-Norteamérica-1/Asia-3. En el primer nodo una bifurcación da origen al linaje Norteamérica-2, en la otra rama vemos que se forma un nodo que a su vez se bifurca dando origen al Linaje Suramérica-3 y en la otra rama se forma otro nodo que presenta una politomía que da origen al Linaje Suramérica/ Norteamérica-4, en la segunda rama se forma un nodo que se bifurca y da origen al Linaje Africa-2, asimismo en la otra rama de este nodo hay una bifurcación que da origen a linaje Asia-2 y en la otra rama ocurre otra bifurcación que da origen a los linajes Tipo Ártico y Africa-1. En la tercera rama de la politomía vemos otro nodo que da origen a los Linajes Asia-1 y Europa/Suramérica-1.

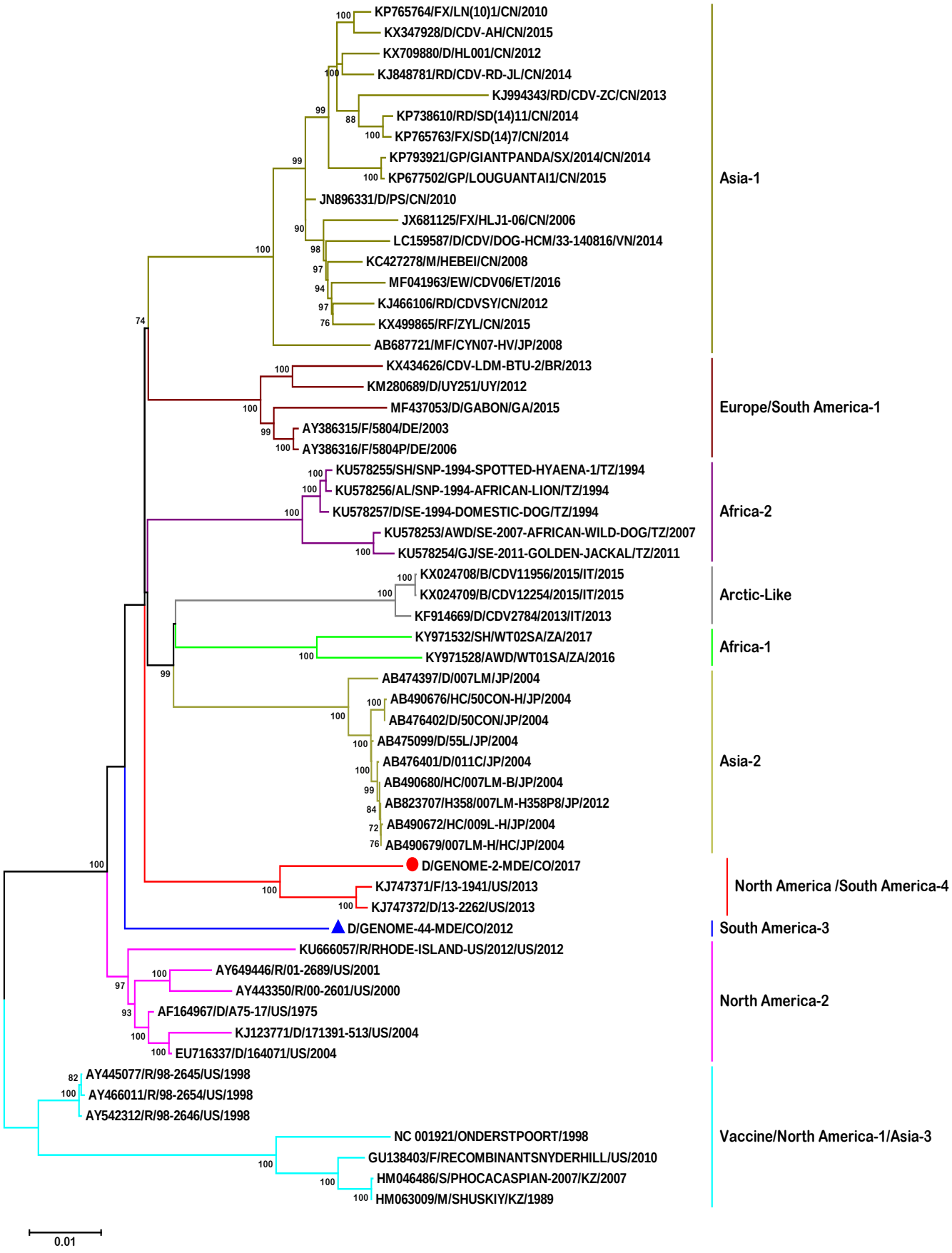
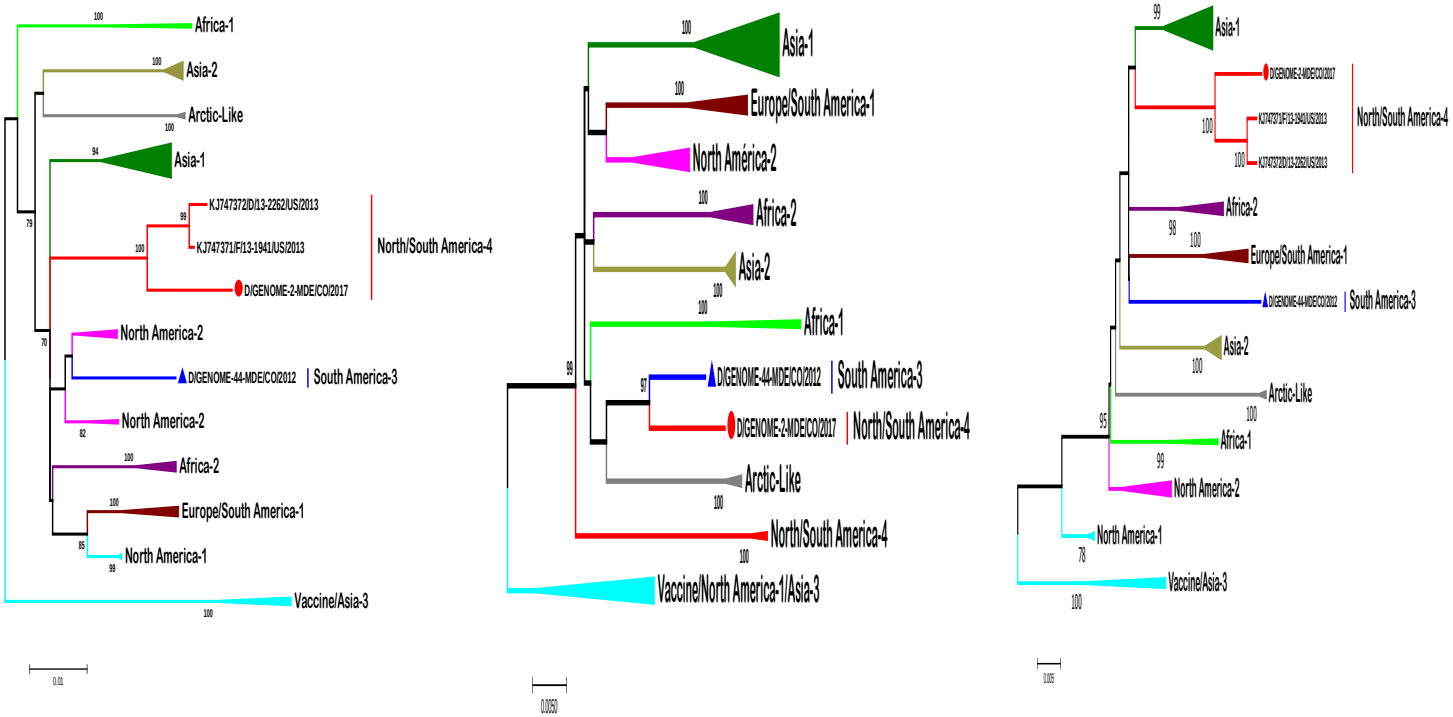

KP765764/FX/LN(10)1/CN/2010
KX347928/D/CDV-AH/CN/2015
KX709880/D/HL001/CN/2012
KJ848781/RD/CDV-RD-JL/CN/2014
KJ994343/RD/CDV-ZC/CN/2013
KP738610/RD/SD(14)11/CN/2014
KP765763/FX/SD(14)7/CN/2014
KP793921/GP/GIANTPANDA/SX/2014/CN/2014
KP677502/GP/LOUGUANTAI1/CN/2015
JN896331/D/PS/CN/2010
JX681125/FX/HLJ1-06/CN/2006
LC159587/D/CDV/DOG-HCM/33-140816/VN/2014
KC427278/M/HEBEI/CN/2008
MF041963/EW/CDV06/ET/2016
KJ466106/RD/CDVSY/CN/2012
KX499865/RF/ZYL/CN/2015
AB687721/MF/CYN07-HV/JP/2008
Asia-1
KX434626/CDV-LDM-BTU-2/BR/2013
KM280689/D/UY251/UY/2012
MF437053/D/GABON/GA/2015
AY386315/F/5804/DE/2003
AY386316/F/5804P/DE/2006
Europe/South America-1
KU578255/SH/SNP-1994-SPOTTED-HYAENA-1/TZ/1994
KU578256/AL/SNP-1994-AFRICAN-LION/TZ/1994
KU578257/D/SE-1994-DOMESTIC-DOG/TZ/1994
KU578253/AWD/SE-2007-AFRICAN-WILD-DOG/TZ/2007
KU578254/GJ/SE-2011-GOLDEN-JACKAL/TZ/2011
Africa-2
KX024708/B/CDV11956/2015/IT/2015
KX024709/B/CDV12254/2015/IT/2015
KF914669/D/CDV2784/2013/IT/2013
Arctic-Like
KY971532/SH/WT02SA/ZA/2017
KY971528/AWD/WT01SA/ZA/2016
Africa-1
AB474397/D/007LM/JP/2004
AB490676/HC/50CON-H/JP/2004
AB476402/D/50CON/JP/2004
AB475099/D/55L/JP/2004
AB476401/D/011C/JP/2004
AB490680/HC/007LM-B/JP/2004
AB823707/H358/007LM-H358P8/JP/2012
AB490672/HC/009L-H/JP/2004
AB490679/007LM-H/HC/JP/2004
Asia-2
D/GENOME-2-MDE/CO/2017
KJ747371/F/13-1941/US/2013
KJ747372/D/13-2262/US/2013
North America /South America-4
D/GENOME-44-MDE/CO/2012
South America-3
KU666057/R/RHODE-ISLAND-US/2012/US/2012
AY649446/R/01-2689/US/2001
AY443350/R/00-2601/US/2000
AF164967/D/A75-17/US/1975
KJ123771/D/171391-513/US/2004
EU716337/D/164071/US/2004
North America-2
AY445077/R/98-2645/US/1998
AY466011/R/98-2654/US/1998
AY542312/R/98-2646/US/1998
NC 001921/ONDERSTPOORT/1998
GU138403/F/RECOMBINANTSNYDERHILL/US/2010
HM046486/S/PHOCACASPIAN-2007/KZ/2007
HM063009/M/SHUSKIY/KZ/1989
Vaccine/North America-1/Asia-3
0.01

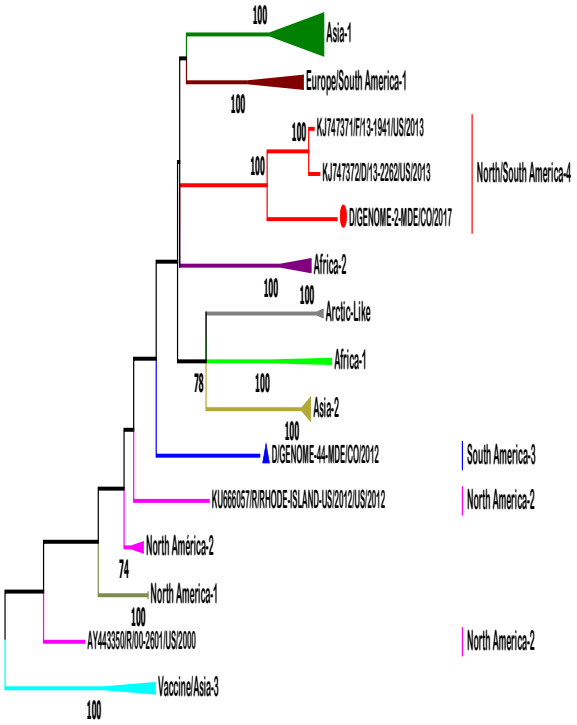
Figura 1. Relaciones filogenéticas entre 58 cepas de CDV basadas en secuencias del genoma. El árbol filogenético se infirió por el método de máxima verosimilitud utilizando 1000 repeticiones. Los números de acceso de GenBank, las especies de las que se obtuvo cada aislado, el nombre de la cepa, el país de origen y el año de aislamiento se indican en las etiquetas de las ramas, si están disponibles. Los números en los nodos son valores Bootstrap para el clado. Abreviaturas de especies animales: AL: African lion *(Panthera leo)*, AWD: African wild dog *(Lycaon pictus)*, B: Badger *(Meles meles)*, BS: Baikal seal *(Pusa sibirica)*, D: Dog *(Canis lupus familiaris)*, F: Ferret *(Mustela putorius furo)*, FX: Fox *(Vulpes urocyon)*, GJ: Golden jackal *(Canis anthus)*, GP: Giant panda *(Ailuropoda melanoleuca)*, HC: Golden hamster *(Mesocricetus auratus)* H358: Human lung cells, M: Mink *(Neovison vison)*, MF: crab-eating macaque *(Macaca fascicularis)*, R: Raccoon *(Procyon lotor)*, RD: Raccoon dog *(Nyctereutes procyonoides)*, RF: Red fox *(Vulpes vulpes)* S: Seal *(Phoca vitulina)*, SH: Spotted hyaena *(Crocuta crocuta)* Abreviatura de países: BR: Brazil, CN: China, CO: Colombia, DE: Germany, ET: Ethiopia, GA: Gabon, IT: Italy, JP: Japan, KZ: Kazakhstan, TZ: Tanzania, US: United States, UY: Uruguay, VN: Vietnam, ZA: South Africa.

Adicionalmente se realizaron árboles filogéneticos de cada gen. Se observa que los genes N, P, M y F permiten en su gran mayoría diferenciar los linajes existentes del CDV. Sin embargo, la topología de los árboles es diferente al compararla entre ellos, con genoma y gen H (Figura 2 a- e). El gen L no permite diferenciar los linajes, presenta 6 bifurcaciones, en la que una presenta una politomía y las secuencias se agrupan sin conservar los linajes ni llevar

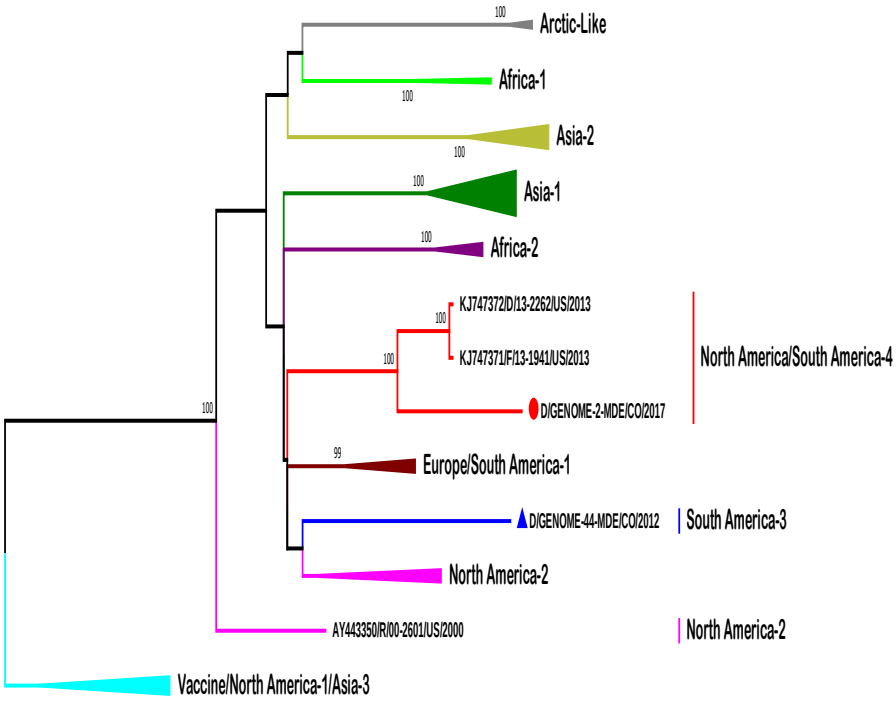
un patrón geográfico, aunque las cepas del linaje Asia-2 en su mayoría están agrupadas en un mismo clado (Figura 2 f)



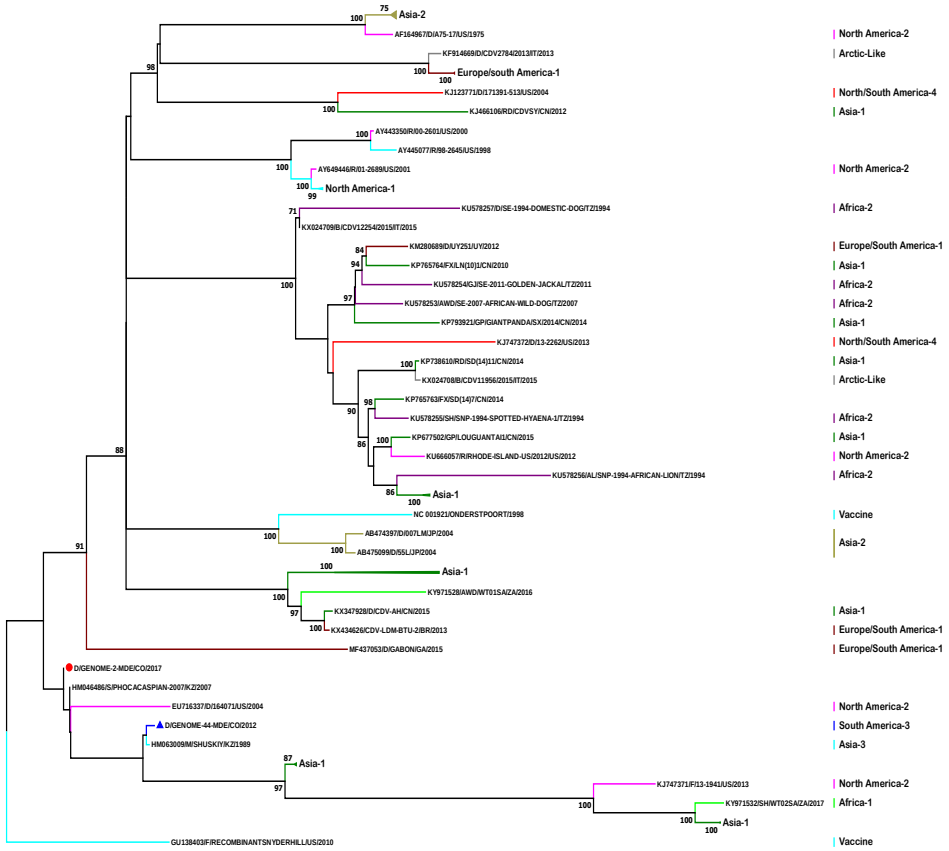
a) Gen N **b) Gen P** **c) Gen M**


Asia-1
Europe/South America-1
KJ747371/F/13-1941/US/2013
KJ747372/D/13-2262/US/2013
D/GENOME-2-MDE/CO/2017
North/South America-4
Africa-2
Arctic-Like
Africa-1
Asia-2
D/GENOME-44-MDE/CO/2012
South America-3
KU666057/R/RHODE-ISLAND-US/2012/US/2012
North America-2
North America-2
North America-1
AY443350/R/00-2601/US/2000
North America-2
Vaccine/Asia-3

d) Gen F


Arctic-Like
Africa-1
Asia-2
Asia-1
Africa-2
KJ747372/D/13-2262/US/2013
KJ747371/F/13-1941/US/2013
D/GENOME-2-MDE/CO/2017
North America/South America-4
Europe/South America-1
D/GENOME-44-MDE/CO/2012
South America-3
North America-2
AY443350/R/00-2601/US/2000
North America-2
Vaccine/North America-1/Asia-3

e) Gen H



f) Gen L

Figura 2. Árboles filogenéticos de cada gen de CDV. Se usaron 58 secuencias de genoma disponibles en GenBank, a las cuales se ubicó el ORF para cada gen y se realizó análisis filogenético por el método de Maxima verosimilitud con bootstrap de 1000 réplicas, a) árbol filogenético gen N; b) árbol filogenético gen P; c) árbol filogenético gen M; d) árbol filogenético gen F; e) árbol filogenético gen H; f) árbol filogenético gen L.

3.3 Análisis de recombinación

Mediante la implementación del programa RDP4 se obtuvieron 2 eventos de recombinación en las cepas circulantes en Colombia: Un evento ubicado en el gen P y un segundo evento ubicado en el gen L, los cuales al ser analizados por el programa Simplot sólo un evento fue

confirmado, por lo cual el otro se descartó. Así el análisis se enfocó en el evento de recombinación ocurrido en el gen P entre las cepas KJ747371/FX/13-1941/US/2013 pariente mayor (Linaje Suramérica/Norteamérica-4) y la cepa D/GENOME-44-MDE/CO/2012 pariente menor (Linaje Suramérica-3), dando origen a la cepa recombinante D/GENOME-2-MDE/CO/2017 (Linaje Suramérica/Norteamérica-4).

La cepa Pariente mayor (KJ747371) es una cepa aislada a partir de muestras de pulmón un zorro del estado de Tennessee (USA) que presentó signos neurológicos antes de la eutanasia (Riley and Wilkes 2015) . Por su parte, la cepa Pariente menor, pertenece a un paciente canino de raza basset hound con síntomas respiratorios y neurológicos y sin antecedentes de salir de su ciudad de nacimiento. La cepa recombinante fue obtenida de un canino de raza pastor alemán con síntomas respiratorios y gastrointestinales.

La región de recombinación fue definida por las metodologías RDP4, GENECONV, BootScan, MaxChi, Chimaera, SIScan,. Todas las metodologías tuvieron un valor de $p<0.05$ (Tabla 2).

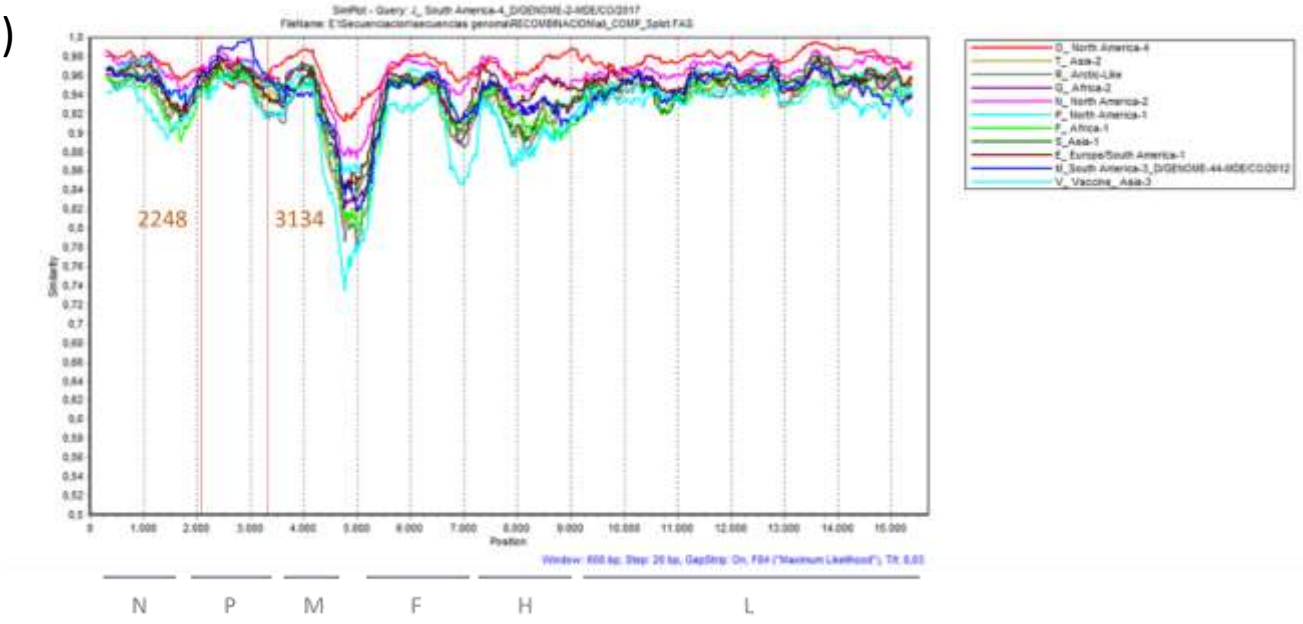
TABLA 2. EVALUACIÓN DE RECOMBINACIÓN HOMÓLOGA EN CDV USANDO RDP4

Método	**# de eventos**	**p-Val**
RDP	1	$4{,}096 \times 10^{-19}$
GENECONV	1	$9{,}527 \times 10^{-15}$
BOOTSCAN	1	$7{,}222 \times 10^{-17}$
MAXCHI	1	$2{,}191 \times 10^{-6}$

CHIMAERA	1	$3,22 \times 10^{-7}$
SISCAN	1	$3,189 \times 10^{-9}$
3SEQ	1	$4,090 \times 10^{-12}$
Inicio punto de quiebre (posición genómica)		2162 99% IC: 2085-2395
Final punto de quiebre (posición genómica)		3300 99% IC: 3136-3331
Tasa de recombinación		$2,69 \times 10^{-4}$
Pariente mayor	KJ747371/F/13-1941/US/2013	Similaridad: 97,30%
pariente menor	D/GENOME-44-MDE/CO/2012	Similaridad: 99,30%

Los puntos de recombinación en el genoma están entre los nucleótidos 2172- 3300 por RDP4, mientras que en Simplot están entre los nucleótidos 2248-3134, los cuales están ubicados en el segmento P que abarca la porción final de la región codificante para la proteína C (Figura 3 y 4).

a)


2248
3134
Position
Similarity
N
P
M
F
H
L

b)

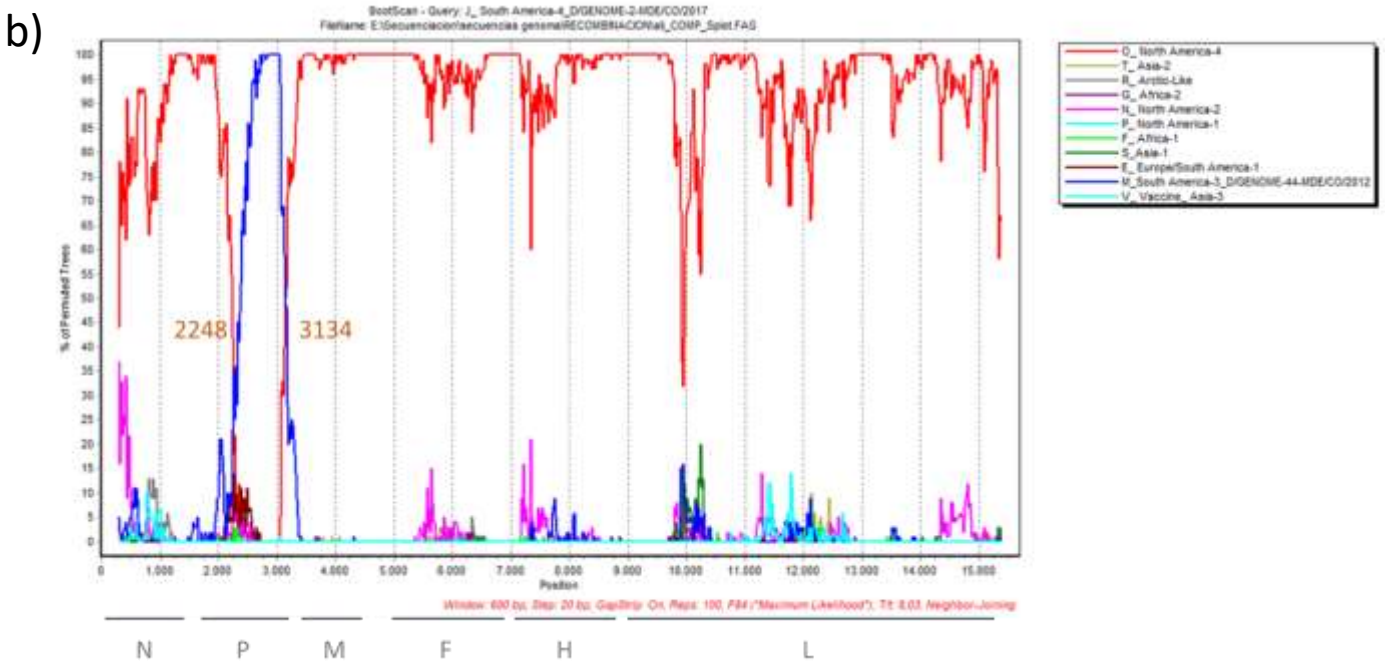
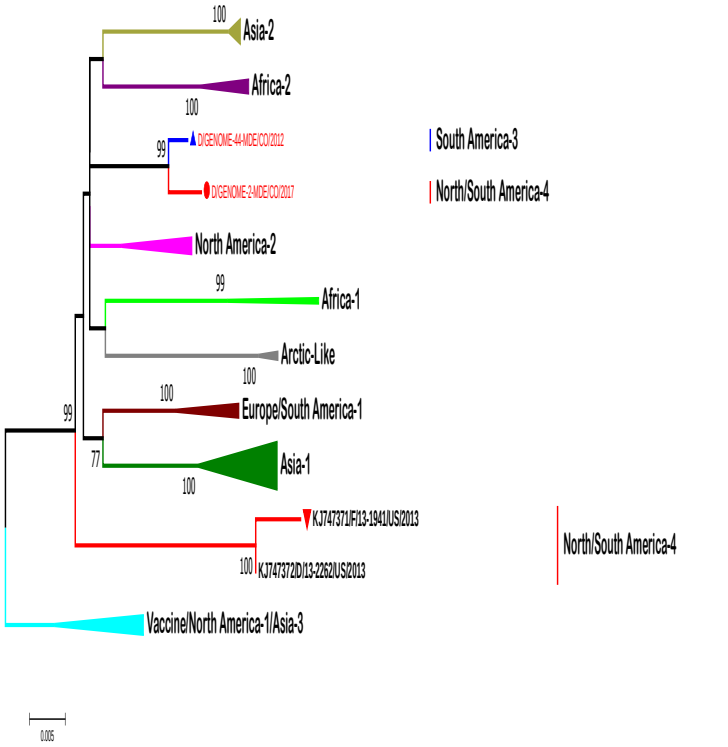
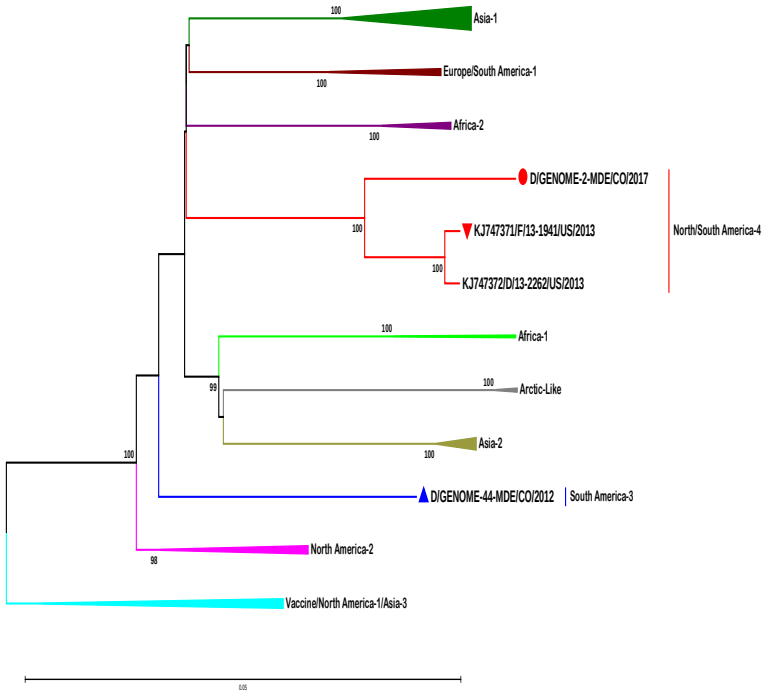

2248
3134
Position
% of Permuted Trees
N
P
M
F
H
L

Figura 3. Evidencia de recombinación homóloga en el genoma de CDV. (a) Análisis de similitud de las secuencias genómicas de los linajes de CDV. En este análisis se usó la secuencia D/GENOME-2-MDE/CO/2017 (Linaje Norte/Suramérica-4) como "query". El eje Y proporciona el porcentaje de identidad con un tamaño de ventana de 600 pb y un tamaño de paso de 20 pb. Las líneas verticales rojas indica las regiones de punto de quiebre de la recombinación. (b) Análisis de escaneo de arranque de D/GENOME-2-MDE/CO/2017 (Linaje Norte/Suramérica-4) y sus secuencias parentales KJ747371/F/13-1941/US/2013 pariente mayor (Linaje Norte/Suramérica-4) y D/GENOME-44-MDE/CO/2012 pariente menor (Linaje Suramérica-3). El eje Y presenta el porcentaje de árboles permutados utilizando una ventana deslizante



a) 2248-3134



b) 1-2247/ 3135-15690

Figura 4. Árbol filogenético de la región recombinante del gen P y no recombinante del genoma. A). Árbol filogenético de los puntos de quiebre del gen N: 2248-3134, se observa que el pariente mayor Linaje Norteamérica-4 secuencia KJ747371/FX/13-1941/US/2013 está en un clado diferente a la cepa recombinante D/GENOME-2-MDE/CO/2017 Linaje Norte/Suramérica-4y esta está en una misma clada con la cepa D/GENOME-44-MDE/CO/2012 pariente menor (Linaje Suramérica-3) .**B)** Árbol filogenético del genoma sin la región recombinante: 1-2247/ 3135-15690, se observa que le cepa recombinante D/GENOME-2-MDE/CO/2017 está en la misma clada con su pariente mayor secuencia KJ747371/FX/13-1941/US/2013 ambas del Linaje Norte/Suramérica-4 , mientras la cepa D/GENOME-44-MDE/CO/2012 pariente menor del Linaje Suramérica-3, está en una única rama.

3.4 Análisis de aminoácidos del CDV

El genoma presenta 5230 aminoácidos de los cuales 3064 presentan variación entre los linajes, de estos 2308 aminoácidos son comunes entre varios linajes y solo 752 aminoácidos autopomórficos.

Al evaluar las diferencias entre la divergencia aminoacídica y nucleotídica de los diferentes genomas del CDV, encontramos una mayor divergencia (7%-9%) en las distancias de aminoácidos con respecto a las distancias de nucleótidos cuando se comparan los linajes (ver tablas 2 y 3). Así, por ejemplo, la divergencia aminoacídica entre los linajes Norteamérica 1 y Suramérica-3 es de 11,2% mientras con la distancia de nucleótidos es de 4.9%. Al comparar

la clasificación de las secuencias de genoma con respecto al gen H, podemos asegurar que los linajes se conservan por ambas metodologías; sin embargo , si comparamos las topologías de los árboles del gen H y genoma de este dataset observamos diferencias por ejemplo con el Linaje Suramérica-3 que en el árbol del genoma está como una rama independiente, pero en el árbol del gen H se agrupa en un mismo clado con el Linaje Norteamérica-2, pero si comparamos las tablas de distancias aminoacídicas de ambos (gen H y genoma) observamos divergencias superiores al 4% en el gen H y del 8 % en genoma, concluyendo que la secuencia clasificada como Linaje Suramérica-3 por ambas metodologías está correctamente clasificada. Si observamos la topología para el Linaje Suramérica/Norteamérica-4 en el árbol del genoma, este surge de una bifurcación, mientras en el árbol del gen H surge de una politomía entre los Linajes Europa/Suramérica-1 y Norteamérica-2/Suramerica-3 (Figuras 1 y 2 - Tabla 3).

Tabla 3. Distancias no corregidas (p) observadas en pares de secuencias de aminoácidos de genoma y gen H en los linajes de CDV.

	SA-4	**NA-4**	**SA-3**	**VAC**	**NA-1**	**ASIA-3**	**NA-2**	**EU/SA-1**	**ARC-L**	**AFR-1**	**AFR-2**	**ASIA-1**	**ASIA-2**
SA-4		**0,003**	**0,004**	**0,005**	**0,005**	**0,005**	**0,004**	**0,004**	**0,005**	**0,005**	**0,005**	**0,005**	**0,005**
		0,007	0,009	0,01	0,011	0,011	0,007	0,008	0,01	0,009	0,009	0,009	0,01
NA-4	**0,061**		**0,004**	**0,005**	**0,004**	**0,005**	**0,004**	**0,004**	**0,005**	**0,004**	**0,005**	**0,004**	**0,005**
	0,031		0,009	0,011	0,011	0,011	0,008	0,008	0,01	0,009	0,009	0,009	0,009
SA-3	**0,123**	**0,121**		**0,005**	**0,004**	**0,005**	**0,004**	**0,004**	**0,005**	**0,004**	**0,005**	**0,004**	**0,004**
	0,06	0,064		0,011	0,012	0,011	0,007	0,008	0,011	0,009	0,01	0,009	0,011
VAC	**0,177**	**0,172**	**0,174**		**0,004**	**0,003**	**0,005**	**0,005**	**0,004**	**0,005**	**0,005**	**0,004**	**0,005**

	0,093	0,095	0,104		0,006	0,006	0,009	0,009	0,011	0,009	0,01	0,01	0,011
NA-1	**0,124**	**0,12**	**0,112**	**0,109**		**0,004**	**0,003**	**0,004**	**0,004**	**0,004**	**0,005**	**0,004**	**0,005**
	0,085	0,088	0,096	0,038		0,007	0,009	0,01	0,011	0,01	0,01	0,01	0,011
ASIA-3	**0,173**	**0,166**	**0,167**	**0,057**	**0,106**		**0,005**	**0,005**	**0,004**	**0,004**	**0,005**	**0,005**	**0,005**
	0,084	0,088	0,096	0,039	0,032		0,01	0,01	0,011	0,01	0,01	0,01	0,011
NA-2	**0,099**	**0,1**	**0,088**	**0,149**	**0,073**	**0,147**		**0,003**	**0,004**	**0,004**	**0,004**	**0,004**	**0,004**
	0,055	0,057	0,056	0,092	0,084	0,083		0,005	0,009	0,008	0,007	0,007	0,009
EU/SA-1	**0,13**	**0,119**	**0,12**	**0,17**	**0,114**	**0,166**	**0,097**		**0,004**	**0,004**	**0,004**	**0,004**	**0,005**
	0,055	0,053	0,056	0,087	0,08	0,077	0,043		0,009	0,008	0,007	0,007	0,009
ARC-L	**0,14**	**0,131**	**0,135**	**0,175**	**0,126**	**0,167**	**0,112**	**0,129**		**0,004**	**0,004**	**0,004**	**0,005**
	0,077	0,071	0,085	0,101	0,092	0,091	0,07	0,066		0,009	0,01	0,01	0,01
AFR-1	**0,137**	**0,129**	**0,129**	**0,166**	**0,126**	**0,163**	**0,11**	**0,13**	**0,128**		**0,004**	**0,004**	**0,004**
	0,062	0,064	0,069	0,092	0,085	0,083	0,062	0,057	0,064		0,009	0,008	0,009
AFR-2	**0,132**	**0,12**	**0,123**	**0,169**	**0,122**	**0,165**	**0,102**	**0,121**	**0,13**	**0,129**		**0,004**	**0,005**
	0,066	0,064	0,073	0,095	0,09	0,089	0,06	0,053	0,08	0,066		0,008	0,01
ASIA-1	**0,131**	**0,118**	**0,122**	**0,172**	**0,122**	**0,167**	**0,102**	**0,118**	**0,134**	**0,133**	**0,123**		**0,005**
	0,065	0,068	0,075	0,097	0,088	0,088	0,061	0,053	0,079	0,067	0,066		0,009
ASIA-2	**0,132**	**0,123**	**0,126**	**0,165**	**0,118**	**0,161**	**0,105**	**0,125**	**0,121**	**0,123**	**0,124**	**0,128**	
	0,071	0,071	0,081	0,102	0,096	0,096	0,07	0,064	0,08	0,068	0,078	0,069	

NA-4: North America-4; SA-4: South America-4; SA-3: South America-3; VAC: Vaccine; NA-1: North America-1, NA-2: North America-2; EU/SA-1: Europe/South America-1; ARC-L:Arctic-Like; AFR-1: Africa-1; AFR-2: Africa-2. En negrita están los valores de las distancias de genoma, debajo están los valores de las distancias del gen H. En color azul se muestra el error estándar estimado, se estableció con un bootstrap de 1000 réplicas

En el análisis de sitios de glicosilación, encontramos los mismos sitios de glicosilación en la proteína H para los linajes Suramérica-3 y Suramérica/Norteamérica-4: 19,149,391, 422 sitios previamente reportados en los demás linajes. En la proteína F en el Linaje Suramérica-3 encontramos los sitios: 62, 108, 141,173y 179 y en el Linaje Suramérica/Norteaméica-4: 141, 173, 179, sitios comunes con los demás linajes.

Para tratar de explicar las posibles fallas vacunales reportadas a nivel mundial, se realizó un análisis comparativo de las secuencias aminoacídicas de las proteínas del CDV de los linajes de campo comparándolas con las secuencias pertenecientes al Linaje America-1 al cual pertenecen las diferentes cepas vacunales, encontrándose múltiples sustituciones, incluso en sitios bajo selección positiva (Tabla 4).

Tabla 4. Sustituciones entre cepas vacunales y cepas de campo

GEN	SUSTITUCIONES
N	T410A, A428T, P448S, H450N, L456F, L456V
P	C40H, C40R, T49A, G50S, N59D, T67A, P97L, P97A, E99D, G106E, E124A, T135A, G137S, R221G, R221E, E233G, L256P, E264G, G270E, N278S, L287P, T426A
M	NO ENCONTRADO
F	K3N, K3E, K7E, P23H, R34Q, A35T, Y48H, D49G, T60I, L74S, N82D, Q88H, K96Q, E103K, P107S, I110T, I110V, S616I, A646T
H	H30Q, T56A, D238Y, R241E, R241G, E247K, D329N, H330Q, M342V, K370Q, G376N, N446D, I506T, D531N, N572D, A586T
L	G139D, T149A, N1705K, S1707P, T1708I, S1712L, H2010Q, H2017Y, S2076F
Los sitios subrayados están bajo selección positiva	

Por otro lado, se trató de establecer sitios que expliquen el salto de la barrera de especies del linaje Suramérica/Norteamérica-4 y Linaje Suramérica-3. Se pudo inferir las sustituciones

presentadas entre la cepa obtenida de un zorro (KJ747371) y las cepas obtenidas de caninos de los linajes Suramérica/Norteamérica-4 y Linaje Suramérica-3, respectivamente. Se describe los aminoácidos únicos en la cepa del zorro en los siguientes sitios en la proteína P: 259I, 431C, proteína C 171G, proteína M 206S, proteína F 621P y proteína H 9D.

Además se comparó el genoma de CDV adaptado a células humanas (H358) (AB823707) y los genomas de los linajes Suramérica/Norteamérica-4 y Suramérica-3 para explorar el potencial zoonótico, para esto se editó la secuencia nucleotídica del gen P adicionando una guanina entre los nucleótidos 751-752 para generar así el ORF de la proteína V (Schuhmann, Pfaller, and Conzelmann 2011), es de aclarar que en los trabajos reportados por (Otsuki, Sekizuka, et al. 2013; Otsuki, Nakatsu, et al. 2013) se habla que la cuasiespecie adaptada a las células humanas tuvo un porcentaje mayor en un nucleótido al compararla con la cuasiespecie aislada del canino por lo cual en la Tabla 5 se enlistan los sitios idénticos al aminoácido "ancestral" caracterizado en la cepa no adaptada a la línea celular H358 y se subrayaron los sitios con las sustituciones ya presentes o derivadas en los linajes de interés. Por lo cual en los dos linajes de interés se encontraron 10 sitios ubicados en las proteínas C, F y L que presentan las sustituciones que permiten la adaptación del CDV a células humanas.

Tabla 5. Sustituciones de CDV adaptado a células Humanas H358.

PROTEINA	SUSTITUCIONES
N	L229V, I296M,E467K
P/V	V133A,M267V
V	Y267C
C	L6W, A27V,T30I, T33A, C44S, R47K, R74L, L89P, T93M, A109V, K146R, K154R,Q172R, P173L

M	T84P, F178L, N206D, L329Q
F	C116Y, R331P
H	D540G, M548T
L	D1748N

Se subrayan los sitios en los cuales la sustitución ya está presente en los linajes Suramérica-3 y Suramérica/Norteamérica-4, en los otros sitios estos dos linajes presentan el a.a "ancestral".

Finalmente se trató de establecer sitios que puedan asociarse a la patogenicidad de los Linajes Norte/Suramérica-4 y Suramérica-3, para esto debemos tener presente que las secuencias Genoma44Med/CO/2012, KJ47372 y KJ747371 fueron obtenidas de animales con sintomatología neurológica y la secuencia Genoma2Med/CO/2017 fue obtenida de un canino con sintomatología respiratoria y gastrointestinal, por lo cual, la sintomatología neurológica fue común en 3 pacientes mientras a sintomatología gastrointestinal varió entre los pacientes, así que describen las sustituciones asociadas al cuadro gastrointestinal (Tabla 6).

Tabla 6. Sustituciones asociadas a síntomas gastrointestinales

PROTEINA	POSICIÓN
N	449V,465G
P	29V, 95R, 100N
C	88G
V	51L, 95R, 100N,143R
F	13V, 19L, 24R, 25S, 40K,45F, 58Q, 64I, 72Y,95P, 99G, 105W,113C,445P, 639R
H	38S,241E,291M,325S,333V,365T,487G 530S

3.5 Análisis de selección positiva

Al realizar el análisis de selección positiva se obtuvieron un total de 38 sitios bajo selección positiva en todo el genoma y un total de 1037 sitios bajo selección negativa. Los genes están bajo selección negativa (Tablas 7 y 8). En la tabla 7 se observa que las proteínas que presentan un mayor número de sitios bajo selección positiva son las proteínas P y V cuya función es la evasión de la respuesta inmune. En la tabla 8 los sitios con mayor tasa de sustituciones no sinóminas dN/dS>10 son P-106, C-3, C-91, C-169, F-53 y H-549.

Tabla 7. Análisis de selección genoma CDV

GEN	# DE AMINOÁCIDOS	# SITIOS SELECCIÓN POSITIVA	# SITIOS SELECCIÓN NEGATIVA	dN/dS
N	524	2	278	0,0249
P	508	9	90	0,05
V	299	9	33	0,177
C	175	8	12	0,128
M	336	2	155	0,005
F	663	7	262	0,02
H	605	1	207	0,006
L	2185	NO ENCONTRADO	NO ENCONTRADO	NO ESTIMADO

Tabla 8. Sitios bajo selección positiva genoma CDV

PROTEINA-SITIO	dN	dS	FACTOR BAYES	dN/Ds

N-134	4,752	0,68	57,7	6,988
N-456	4,608	0,627	67,4	7,349
P-72	5,418	0,728	33,78	7,442
P-106	7,585	0,718	83,17	10,564
P-143	6,457	0,741	58,032	8,714
P-148	6,17	0,6859	67,527	8,995
P-195	6,013	0,681	58,412	8,830
P-221	6,123	0,668	63,472	9,166
P-237	4,641	0,588	42,048	7,893
P-287	4,196	0,587	47,292	7,148
P-296	4,047	0,68	24,756	5,951
V-72	4,465	0,626	24,267	7,133
V-90	2,82	0,587	11,454	4,804
V-106	5,924	0,634	45,958	9,344
V-143	4,106	0,683	21,431	6,012
V-148	5,739	0,578	59,668	9,929
V-195	5,741	0,613	55,469	9,365
V-221	3,924	0,622	20,431	6,309
V-235	3,493	0,619	18,332	5,643
V-237	2,835	0,576	11,792	4,922
C-3	9,158	0,538	822,524	17,022
C-37	3,789	0,661	11,962	5,732
C-45	5,164	0,625	30,715	8,262
C-86	4,998	0,661	16,887	7,561
C-91	9,03	0,654	123,251	13,807
C-99	5,901	1,052	17,952	5,609
C-169	7,423	0,65	57,385	11,420
C-171	4,37	0,667	19,927	6,552
M-9	4,921	0,591	32,626	8,327
M-294	5,606	0,64	59,512	8,759
F-21	7,141	0,781	60	9,143
F-53	7,199	0,695	87,613	10,358

F-72	6,631	0,709	62,9	9,353
F-98	6,919	1,365	26,563	5,069
F-99	6,608	0,796	50,091	8,302
F-101	6,828	0,817	42,661	8,357
F-102	6,105	0,714	49,482	8,550
H-549	10,547	1,037	272,214	10,171

3.6 Reloj molecular CDV

Se obtuvo la tasa de sustitución media y el tiempo l ancestro común más reciente (tMRCA)para cada gen; observamos que el gen P presenta la menor tasa de sustitución mientras que el gen H presenta la mayor tasa de sustitución y si promediamos el tMRCA de cada gen encontramos un tMRCA 1889 con un intervalo entre 1835-1931, ver tabla 9

Tabla 9. Modelo evolutivo genoma CDV

GEN	**MODELO SUSTITUCIÓN**	**TASA EVOLUTIVA**		**tMRCA**	
		MEDIA	Intervalo HDP 95%	MEDIA	Intervalo HDP 95%
N	T92+G	$3{,}73 \times 10^{-4}$	$2{,}71 \times 10^{-4}$ - $4{,}72 \times 10^{-4}$	1931	1905-1954
P	TN93+G	$2{,}65 \times 10^{-4}$	$1{,}69 \times 10^{-4}$ - $3{,}61 \times 10^{-4}$	1888	1838-1927
M	T92+G	$3{,}29 \times 10^{-4}$	$1{,}91 \times 10^{-4}$- $4{,}72 \times 10^{-4}$	1892	1835-1938
F	T92+G	$3{,}25 \times 10^{-4}$	$2{,}06 \times 10^{-4}$- $4{,}41 \times 10^{-4}$	1835	1766- 1891
H	T92+G	$4{,}99 \times 10^{-4}$	$3{,}87 \times 10^{-4}$- $6{,}13 \times 10^{-4}$	1903	1876- 1928
L	GTR+G	NO CALCULADA	NO CALCULADA	NO CALCULADA	NO CALCULADA

4. Discusión

En el presente trabajo obtuvimos dos genomas completos del CDV usando el método de secuenciación Sanger y se trabajó con el RNA total extraído directamente de muestra clínica (secreción ocular y nasal), sin realizar previo aislamiento viral, con lo cual, podemos asegurar que, con la estrategia de trabajar con 15 fragmentos de 1200 nucleótidos y superpuestos, los resultados cuentan con buen soporte tal como se observa en los árboles filogenéticos (Figuras 1 y 2).

Si bien la clasificación filogenética del CDV se ha establecido usando el gen H ahora con la disponibilidad de genomas de los diferentes linajes podemos verificar esta clasificación, y según nuestros resultados los linajes se conservan aunque la topología de los árboles es diferente, debido probablemente a las altas tasas evolutivas que presenta el gen H en comparación con los demás genes (Panzera et al. 2015). Aunque previos realizados con genoma completo no hacen este tipo de análisis (Marcacci et al. 2014; Yuan et al. 2017; Maganga et al. 2018) en nuestro estudio queríamos confirmar si los linajes reportados en este trabajo y los reportados previamente (Espinal, Díaz, and Ruiz-Saenz 2014) se conservaban a nivel de genoma. Igualmente al evaluar los árboles filogenéticos de cada gen se puede observar que se conserva la distribución de linajes previamene determinada en la mayoría de ellos (Figura 2) excepto el gen L, que si bien se espera que este gen sea el más conservado y por ende el filograma presente pocos clados, en este estudio observamos lo contrario

posiblemente se deba a los eventos de recombinación que se han reportado en este gen (Yuan et al. 2017).

La demostrada cocirculación de dos linajes de CDV en Medellín, abre la posibilidad de la existencia de pacientes co-infectados con los dos linajes circulantes. Basados en esta premisa se exploró la recombinación entre estos dos linajes, encontrando la presencia de recombinación homóloga en el gen P (Figuras 3 y 4). Aunque la recombinación es un mecanismo poco frecuente en los virus RNA en sentido negativo (Chare, Gould, and Homes 2003), en el CDV ya se ha sido previamente reportada entre cepas vacunales y cepas silvestres, así (Yuan et al. 2017) trabajan con seis linajes de CDV pero enfocados en los circulantes en Asia, coincidiendo con (Budaszewski da Fontoura et al. 2016) que encontraron recombinación entre cepas vacunales y cepas silvestres no solo en Asia sino en Europa. El presente trabajo encontró las mismas recombinaciones previamente reportadas; sin embargo, solo nos enfocamos en los linajes circulando en Colombia los cuales nunca había sido reportados.

La co-infección en un paciente con dos los linajes silvestres de CDV circulantes en una misma región es un hallazgo singular, en este estudio se presentó el evento de recombinación entre el posible pariente mayor de una cepa obtenida de zorro de los Estados Unidos y el pariente menor es una cepa obtenida de un perro de Medellín y el potencial recombinante fue una cepa obtenida de un perro de Medellín; la recombinación en el gen P puede aumentar el número de sitios selección positiva obteniendo falsos positivos (Pérez-Losada et al. 2015), sin embargo para este data set, se realizaron árboles filogenéticos para cada gen y sólo se

evidenció recombinación en el gen P para los linajes Suramérica-3 y Suramérica/Norteamérica-4, por lo que se asume que para el resto de genes estos sitios bajo selección positiva son válidos, aunque nosotros encontramos sitios bajo selección positiva en el gen P (148, 195,221 y 287). Coincidiendo con resultados recientemente publicados para otros linajes (Yuan et al. 2017), podemos inferir que los dos tipos de mecanismos, selección y recombinación, están involucrados en la evolución del virus.

Si analizamos los sitios de selección positiva en el gen P de los linajes Suramérica-3, Norte/Suramérica-4 con las cepas vacunales vemos que en el sitio 106 hay una sustitución de una glicina por ácido glutámico en el linaje Norte/Suramérica-4, en el sitio 221 hay una sustitución de arginina por glicina en ambos linajes, en el sitio 278 hay una sustitución de asparagina por serina en ambos linajes; estas sustituciones están presentes en estos sitios sometidos a selección positiva, además estos sitios están próximos o dentro de epítopes de presentación a linfocitos B reportados por (S. Li et al. 2018) y debido a la alta divergencia en estos epítopes los anticuerpos monoclonales encontrados en dicho estudio pueden diferenciar entre cepas vacunales y silvestres (S. Li et al. 2018). Tomando en conjunto estos resultados, es posible postular que cambios en este gen también contribuyen a la baja o nula producción de anticuerpos vacunales capaces de neutralizar las cepas silvestres (respuesta humoral), como se ha reportado para las cepas caracterizadas en Estados Unidos pertenecientes al Linaje Suramérica/Norteamérica-4 o incluso puede ser una de las explicaciones a cepas de inmunoescape (Zhao et al. 2014; Anis, Holford, et al. 2018).

Respecto a la patogénesis, la proteína V está relacionada con suprimir la respuesta inmune innata al inhibir la inducción de los interferones tipo I y II y la actividad del complejo NF-

κβ al interactuar el sitio Y110 con las moléculas STAT1 y la región W240-W250 al unirse con STAT2, (Schuhmann, Pfaller, and Conzelmann 2011; Chinnakannan, Nanda, and Baron 2013; Svitek et al. 2013). Además la proteína V inhibe la vía de señalización Mda5 por la unión de los sitios R233-E235 (Svitek et al. 2013). Así, en sarampión se determinó que la proteína C suprime la vía de señalización del INFγ al inhibir la dimerización de la proteína STAT1 fosforilada, encontrándose los sitios 13,25.39,44,78,104 y 168 relacionados en esta actividad (Yokota, Okabayashi, and Fujii 2011). Al comparar los sitios bajo selección positiva de estas proteínas y los involucrados en la evasión de la respuesta inmune encontramos sitios comunes o muy cercanos lo que da luces que esta proteína influye en el aumento de la aptitud biológica (fitness) para adaptarse a ambientes determinados como células nerviosas, células de diferentes especies o incluso humanas como ha sido previamente reportado (Otsuki, Nakatsu, et al. 2013; Nikolin et al. 2016).

El genoma de CDV presenta una mayor divergencia entre sus aminoácidos con respecto a sus nucleótidos esto es debido a que se están presentando mayor número de sustituciones no sinónimas, lo cual tiene como principio la carencia de autocorrección de la polimerasa viral de los virus RNA (MacLachlan and Dubovi 2011). Por dicha razón, estas sustituciones se han tratado de relacionar con fallas vacunales, salto de la barrera de especie y virulencia. Nuestro estudio permitió demostrar la presencia de sustituciones en sitios bajo selección positiva que están vinculados con estos fenómenos y además están involucrados en la evasión de la respuesta inmune innata y humoral.

Se evidenció en la Nucleoproteína un épitope (sitios 444-455) de presentación a linfocitos B (respuesta humoral), el cual ha sido descrito como altamente variable y asociado a cepas virulentas (Yi et al. 2017). Nuestros resultados coinciden en encontrar sustituciones y presión de selección positiva en dicho epítope en los virus de CDV Linajes Suramérica-3 y Suarmérica/Norteamérica-4, lo cual podría potencialmente explicar fallas vacunales. Asimismo, en la proteína de Fusión se ha descrito un mecanismo de resistencia a antivirales involucrando las sustituciones I564C,V571C,G572C y L575C (Kalbermatter et al. 2018), en nuestros linajes se presentan los mismos aminoácidos ancestrales I564, V571, G572, que si bien en este estudio estos sitios no están bajo presión de selección deben estudiarse en los linajes de interés para la implementación de tratamiento antiviral en nuestro medio.

Se deben considerar los sitios hallados bajo selección positiva prestando mayor atención a los sitios P-106, C-3, C-91, C-169, F-53 y H-549, los cuales están ubicados en proteínas relacionadas con la evasión de la respuesta inmune innata, transmisión y diseminación a otros hospederos y salto de la barrera de especies, respectivamente (Mccarthy, Shaw, and Goodman 2007; Yokota, Okabayashi, and Fujii 2011; Bevan Sawatsky, Cattaneo, and von Messling 2018), con el fin de realizar estudios *in vitro* para asociarlos a inmunopatogénesis en los dos linajes caracterizados y dilucidar mecanismos de evasión de la respuesta inmune, adaptación a diferentes hospederos y virulencia.

Nosotros encontramos que las tasas de sustitución por sitio por año de cada gen son altas ($2.65 \times 10^{-4} – 4.99 \times 10^{-4}$), esto debido a que el CDV es un virus RNA monocatenario, con genoma de tamaño pequeño y además presenta recombinación, características que se han observado en los virus que tienen las tasas evolutivas más altas (Sanjuán and Domingo-Calap 2016), siendo el gen H con la más alta tasa evolutiva coincidiendo con (Panzera et al. 2015)

quien considera que además de las altas tasas evolutivas, el CDV presenta rápida evolución, debido a al flujo genético intenso soportado en las rutas de migración del CDV, y la influencia posterior de factores ecológicos particulares y presiones selectivas locales (Panzera et al. 2015; Nikolin et al. 2016).

En conclusión, nosotros describimos la co-circulación de dos linajes de CDV en una región poco extensa, lo cual posibilita la co-infección en pacientes permitiendo la recombinación homóloga como uno de los mecanismos evolutivos, además caracterizamos sustituciones relacionados con fallas vacunales, salto de la barrera de especie y virulencia, los cuales se deben tener presentes para dilucidar la inmunopatogenia del CDV en nuestra región. Asimismo, confirmamos que el CDV múltiples sitios con selección positiva y recombinación, mecanismos involucrados en la evolución de este agente infeccioso.

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CONCLUSIONES GENERALES

1. Se describe la circulación geográfica de Linaje Suramérica-3 en tres regiones de Colombia: Medellín, Bucaramanga y Bogotá

2. Se reporta la circulación de un nuevo linaje nunca descrito en Colombia, que está relacionado filogenéticamente con cepas circulantes en Ecuador y en Estados Unidos, en este último reportado circulando en fauna silvestre y perros.

3. Se determinaron sustituciones en los genes de CDV relacionadas con la falla vacunal, salto de la barrera de especie y virulencia en los linajes circulantes en Colombia.

4. Se encontraron sitios bajo presión selectiva positiva, relacionados con falla vacunal y evasión de la respuesta inmune.

5. Se logró secuenciar dos genomas del CDV circulando en Colombia.

6. Se describe recombinación homóloga por primera vez en dos linajes de cepas silvestres co-circulando en una área geográfica pequeña.

RECOMENDACIONES GENERALES

1. Caracterizar filogenéticamente el CDV en otras regiones de Colombia: regiones norte y oriente, podría dar evidencias de co-circulación del Linaje Suramérica-3 con otros linajes reportados en Suramérica.
2. Realizar estudios de neutralización de anticuerpos vacunales frente a las cepas circulantes en Colombia, para evidenciar el nivel de protección conferido por las vacunas actuales.
3. Secuenciar genoma completo de otras secuencias por métodos de última generación, para obtener más información de muestras clínicas.
4. Realizar estudios de inmunopatogenia evaluando los sitios bajo selección positiva hallados en este estudio.
5. Secuenciar genoma completo de las cepas que se sospecha son cepas de escape inmune.

ANEXOS

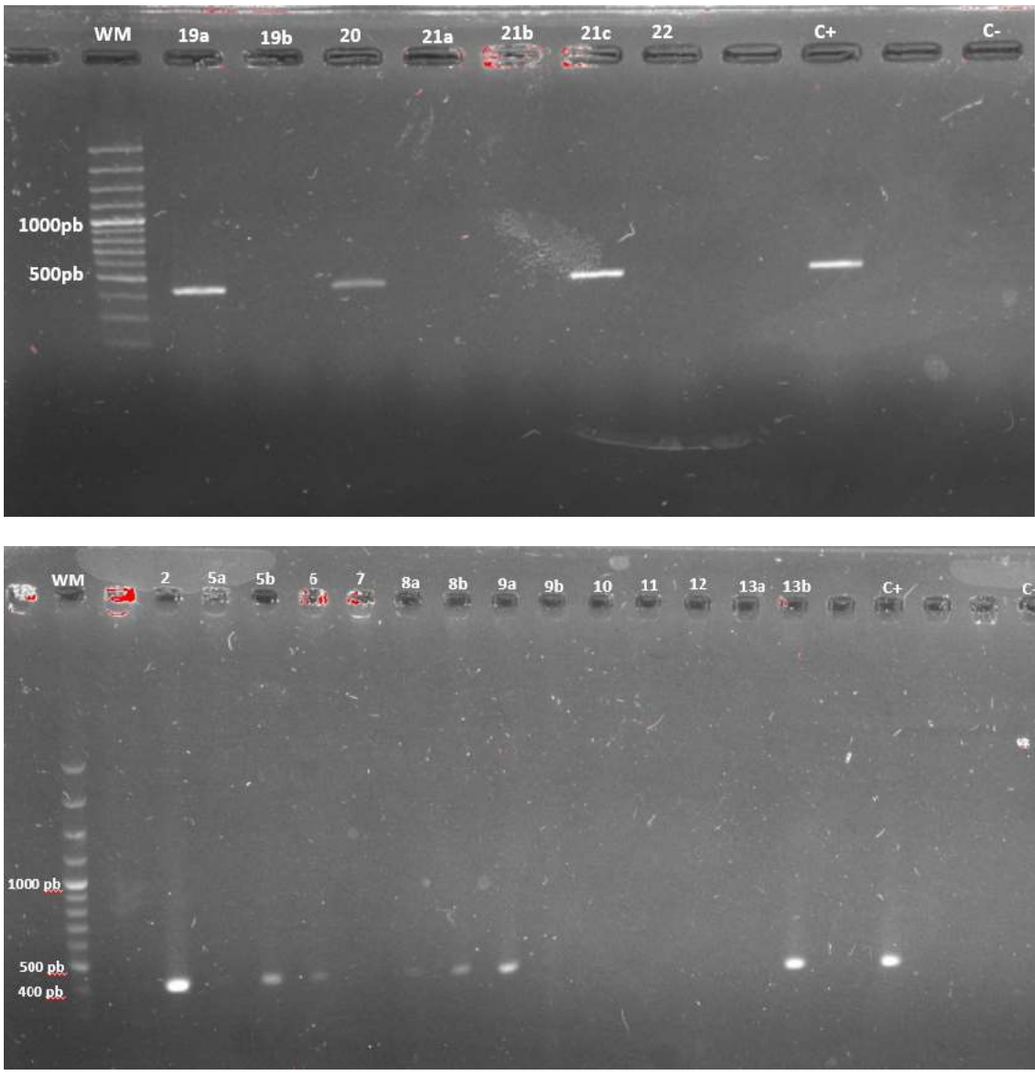


Figura1. Electroforesis en gel agarosa al 1.5% del producto amplificado por PCR del segmento del gen de la Fosfoproteína (429pb). WM: Marcador de peso molecular. C+: Control positivo. C- Control negativo. Resto de pozos muestras de pacientes.

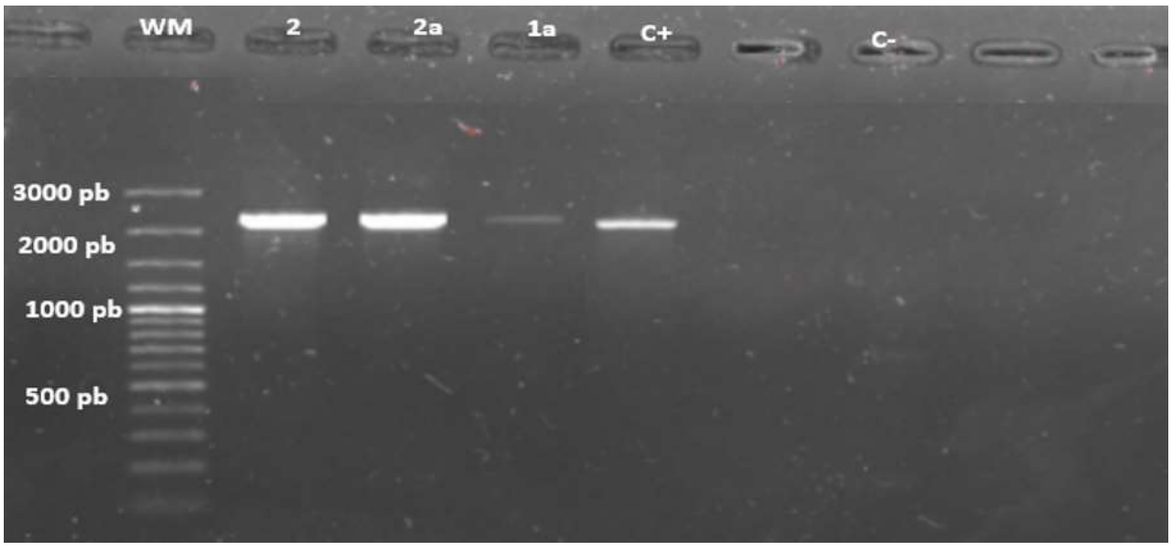
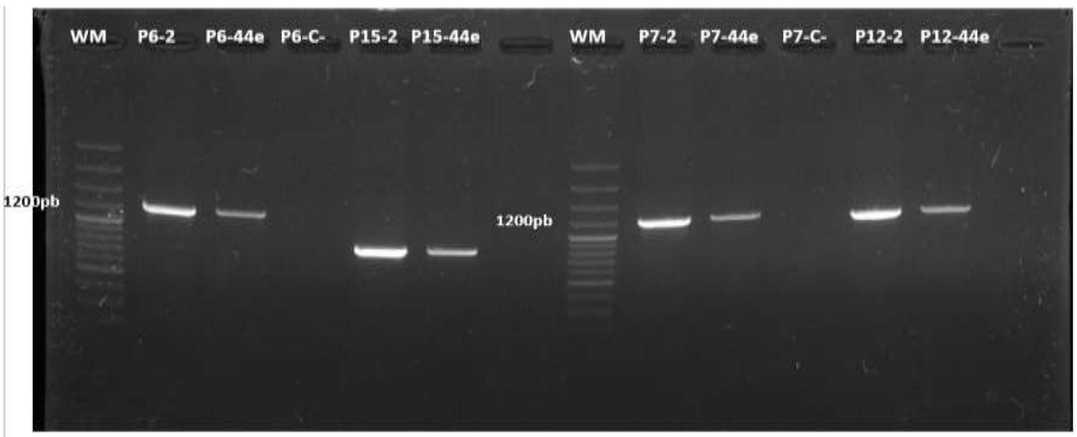
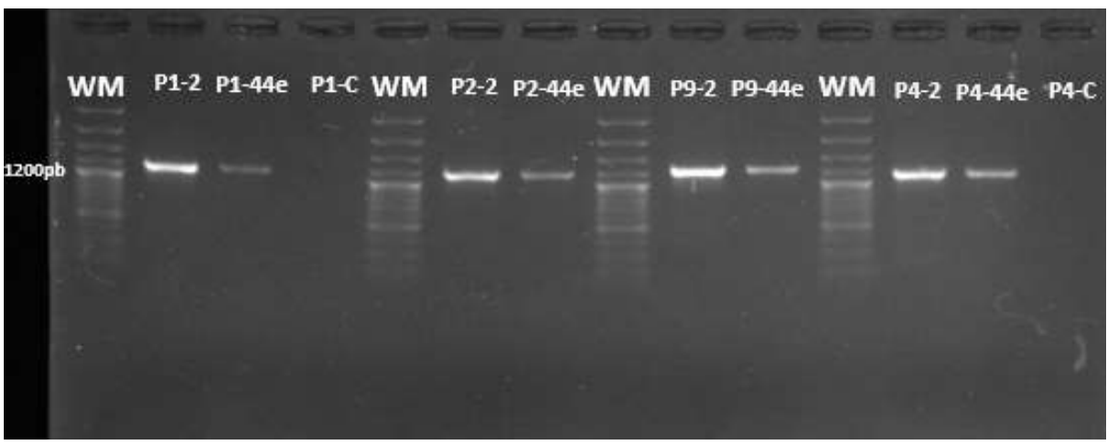


Figura 2. Electroforesis en gel agarosa al 1.5% del producto amplificado por PCR del gen Hemaglutinina de 3 pacientes. (2100pb)





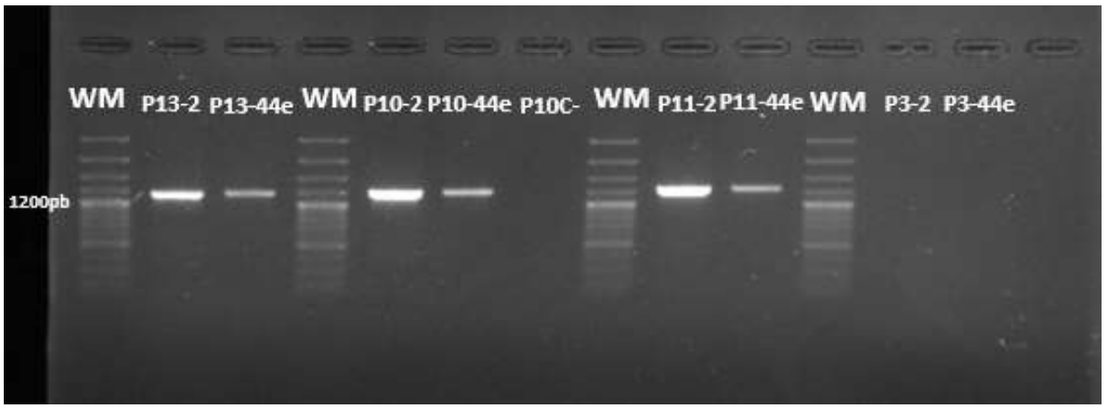


Figura 3. Electroforesis en gel agarosa al 1.5% del producto amplificado por PCR de los segmentos de genoma del CDV de dos pacientes.