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International Journal of Current Research
Vol. 10, Issue, 08, pp.72402-72407, August, 2018

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

PHYLOGENETIC ANALYSIS OF CANINE DISTEMPER VIRUS DETECTED IN CHILE

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ARTICLE INFO

Article History:
Received 27th May, 2018
Received in revised form
20th June, 2018
Accepted 06th July, 2018
Published online 30th August, 2018

Key Words:
Canine distemper,
Hemagglutinin,
Vaccine.

ABSTRACT

Emerging infectious diseases constitute one of the biggest problems facing human and animal health, and biodiversity conservation. Canine distemper virus has been strongly called attention in this regard, since it possesses a high prevalence in the canine population worldwide. Canine distemper is a systemic viral disease, highly contagious, one of the major causes of death in domestic dogs and other carnivores. In recent years, the incidence of canine distemper seems to have increased, documenting the occurrence of new and unusual strains. The reasons that explain these changes and their impact on the epidemiology of the virus still unknown. Canine distemper virus appears genetically heterogeneous, markedly in the hemagglutinin protein, which shows geographic patterns of diversification that are useful to monitoring the molecular epidemiology of canine distemper virus.
In this work was detected hemagglutinin gene (H gene) of canine distemper virus using the Reverse Transcription PCR method, was sequenced the amplified DNA fragment and this nucleotide sequence was included in the phylogenetic analysis for the gene H using known sequences and official of canine distemper virus, including vaccine strains used for the prevention of disease (Genbank®). The results show that in Chile would be at least two of the lineages known for canine distemper virus: Europe and America-1.

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Citation: Salas, V., Pizarro, J. and Navarro, C. 2018. "Phylogenetic analysis of canine distemper virus detected in Chile", *International Journal of Current Research*, 10, (08), 72402-72407.

INTRODUCTION

The canine distemper virus (CDV) is classified in to *Morbillivirus* genus of the family *Paramixoviridae*, from the *Mononegavirales* order. This genus also includes the measles virus, the rinderpest virus, the plague of small ruminants, the distemper of seals and the morbillivirus of cetaceans. Its genome consists of a single chain of unsegmented RNA of negative polarity, which codes for M membrane protein, two types of glycoproteins (hemagglutinin H and fusion protein F), two proteins associated with transcriptase (phosphoprotein P and polymerase L) and for the nucleocapsid N protein that encapsulates the viral RNA (Martella *et al.,* 2008). The F and H proteins induce the production of neutralizing antibodies synthesized by the host's immune system (Appel and Summers, 1995). Some comparative studies among strains of CDV reveal that the H gene is subject to greater genetic and antigenic variations than the other CDV genes: its amino acid sequence varies approximately by 10% between the different CDV strains (Martella *et al.,* 2008) and despite differences antigenic among strains of CDV demonstrated serologically, only one serotype is accepted.

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DOI: https://doi.org/10.24941/ijcr.31909.08.2018

However, there are considerable differences regarding its pathogenicity (Appel and Summers, 1995). The range of CDV hosts includes all species of the Canidae family (dog, dingo, fox, coyote, jackal, wolf), Procionidae (raccoon, coati, red panda), mustelidae (weasel, ferret, mink, skunk, badger, ermine, marten, otter), the big cats of the family Felidae (lion, leopard, cheetah, tiger) and the peccary collar. The numerous reports of distemper outbreaks in lions of the Serengeti National Park of Tanzania and cases in Chinese leopards (*Panthera pardus japonensis*) and other big cats in zoos, have graphically confirmed the ability of the virus to invade new hosts (Maclachland and Dubovi, 2011; Meli *et al*., 2010).

Pathogenesis of the Disease: Young dogs are more susceptible than adults to CDV infection, showing the highest susceptibility within four to six months of life, after the puppies have lost their maternal antibodies (Murphy *et al.*, 1999.). Virus transmission occurs mainly through direct contact, secretions or aerosols, as it is not stable in the environment. CDV is eliminated by all secretions and excretions, starting on the fifth day post infection before the clinical signs begin, and continues to be eliminated even for weeks (Maclachland and Dubovi, 2011). If the immune response is adequate, the neutralizing antibodies will reach adequate levels and eliminate the virus from the tissues, recovering the animal completely. If the immune response is

weak or delayed, the virus is able to reach the epithelial tissues and cause a severe multisystem disease that includes respiratory, digestive and later, central nervous system signs (Martella *et al.,* 2008). The neurological signs may be caused directly by the virus or may be the result of the immune response against the CNS. In the latter case, dogs have a level of intermediate immunity, and the subsequent commitment of the CNS can develop months or years later (Greene, 2008). Currently there is no effective treatment against Canine Distemper (CD), consisting only of support care and antibiotics to prevent bacterial infections secondary, frequent in immunocompromised animals (Martella *et al.,* 2008). In dogs that survive subclinical or subacute infection the initial signs disappear, but the virus persists for long periods in neuronal tissue, uvea or in urothelium and in some areas of the skin (example: hyperkeratosis of the footpad or nose). Neurological signs will continue to evolve progressively, albeit discontinuously. Some dogs may recover, however, compulsive involuntary movements, such as myoclonus or ataxia, will tend to persist (Martella *et al.,* 2008). Finally, the dog dies due to massive damage to the CNS derived from the exaggerated inflammatory process (Céspedes *et al*., 2010).

Diagnostic Methods

DC should always be considered within prediagnostics in front of any puppy with febrile conditions and multisystem symptoms (Martella *et al.,* 2008). Although the systemic disease is easy to recognize, the neurological form can be more complicated. Clinical pathological changes include inclusion bodies in erythrocytes and mononuclear cytology of cerebral spinal fluid. Chest x-ray can help demonstrate viral pneumonia with secondary bacterial infection (Greene, 2008). However, a laboratory diagnosis is necessary to exclude other diseases that have similar clinical manifestations (Maclachland and Dubovi, 2011). Thus, it is possible to include several diagnostic techniques such as immunofluorescence (Maclachland and Dubovi, 2011;Martella *et al.,* 2008)., analysis of cerebrospinal fluid (LCE (Appel and Summers, 1995), ELISA for the detection of specific IgM against CDV (Appel and Summers, 1995), viral isolation (Martella *et al.,* 2008) and molecular techniques such as PCR (Polymerase Chain Reaction) after reverse transcription of the viral genome (RT- PCR). Using these techniques, it has been possible to recognize at world level the existence of at least fourteendifferent canine distemper lines, based on the sequencing analysis of the H gene (Asia 1, Asia 2, America 1, America 2, Arctic, European, Wild European and South African) and probably new and unusual strains will be identified in the future (Woma *et al.,* 2009; Maclachland and Dubovi, 2011; Ke, 2015). Example of the above, is the work in which a ninth lineage would be described in Mexico (Gamiz *et al*., 2011). The results using this technique are promising for the diagnosis of neuronal distemper when the virus is found in tissues and fluids. However, it should be borne in mind that it could deliver false positive results in dogs that come in contact with low levels of the virus and recover after a mild infection or in vaccinated dogs (Martella *et al.,* 2008). In this context, this report proposes as its main objective to know the existing CDV lineages would be present in Chile, through the partial sequencing of the CDV H gene after the PCR test. Apparently and according to the literature consulted this study would be the first contribution in relation to understanding probably why vaccinated dogs get sick and die.

MATERIALS AND METHODS

Clinical and vaccine samples: During the years 2011 and 2012 forty-two samples of peripheral blood of domestic dogs were obtained in different veterinary clinics of Santiago de Chile with a wide range of symptomatology compatible with DC, without restriction of race, age, or sex. Two CDV strains from commercial vaccines (Lederle and Onderstepoort) were used as a reference virus.

Obtaining Viral RNA: RNA extraction was carried out by means of an extraction kit (Trizol LS, Invitrogen ©). Briefly, 250 uL of serum was mixed with 750 uL of trizol reagent. It was left at room temperature (RT) for five minutes and subsequently, 0.2 mL was added of chloroform. It was mixed vigorously for fifteen seconds and left to stand at room temperature for five minutes. Subsequently, it was centrifuged at 7,000 × g for fifteen minutes and the aqueous phase was transferred to a clean tube with a volume of isopropanol. It was left at room temperature for ten minutes and centrifuged at 7,000 × g for ten minutes. Then the supernatant was removed, washed three times with ethanol (1 mL ethanol 75%), vortexed for fifteen seconds and centrifuged at 2,000 × g for five minutes. The supernatant was removed, the precipitate was dried under vacuum for ten minutes and resuspended in 100 uL of sterile, nuclease-free water. Finally, the RNA was incubated at 55-60 ° C for ten minutes and stored at -20 ° C until the RT-PCR was performed.

RT-PCR: For the implementation of this technique an Apollo thermocycler (CLP, USA) was used 96 wells of 0.2 mL and a protocol that involves temperatures and times for each stage, as well as the number of cycles necessary for the gene to be detected. The "SuperScript one step RT-PCR with platinum Taq" kit (Invitrogen®) was used and the protocol contemplated an incubation at 50 ° C for forty minutes and another at 94 ° C for two minutes. In the PCR reaction the CDV1 primers were used: 5'-GTCCTT CTCATCCTACTGG-3’ and CDV2: 5'-ACACTCCGTCTGAGATAGC- 3’ and a protocol that includes 35 cycles (94º C for one minute, 50º C for two minutes, two minutes at 72º C) and a final extension at 72º C for two minutes. This protocol allowed obtaining a DNA fragment of around 560 base pairs (bp) (Pardo *et al.,* 2005). All samples were analyzed in duplicate for confirmation of results. The visualization of the amplified product was performed in 2% agarose gel electrophoresis (Winkler®) in Tris acetate EDTA (TAE) buffer (Fermentas®) and subsequent incubation with ethidium bromide (0.5 µg / ml) (Fermelo®). 5µL of the PCR product was taken, and mixed with 1µL of a commercial loading product, 6X Mass Ruler Loading Dye Solution (Fermentas®), to verify the progress of migration of the DNA bands. Electrophoresis was performed at 90V for 90 minutes. As a molecular size marker, Hyperladder I (Bioline®) was used, which contains fragments of DNA between 50 and 1000 base pairs. With this product the size of the amplified fragments was compared. At the end of the electrophoresis, the DNA bands were visualized in a transilluminator of UV light (Transiluminator UVP®) and photographed with a digital camera and a suitable filter.

Sequencing and phylogenetic analysis of the amplified product

The amplified DNA fragment was sent to the sequencing center of the company Genytec® fulfilling its requirements.

The sequences obtained were aligned using the Clustal Ω open access program (Thompson *et al.,* 1994) to obtain a consensus sequence and subsequently the percentage of nucleotide identity was obtained with respect to CDV sequences stored in the GenBank® using the open access program BLAST. The phylogenetic analysis of the nucleotide sequences obtained was carried out using the MEGA program of bioinformatic analysis (Kumar *et al*., 1994) in which the nucleotide sequences were aligned using the Clustal Ω program (Thompson *et al.,* 1994). The genetic distances between them were calculated using Kimura's two-parameter method and the phylogenetic trees were constructed using the "neihbor-joining" method (Kimura *et al*., 1994). The robustness of the phylogenetic tree obtained was determined by "bootstrap" analysis of a thousand replicas.

RESULTS

Clinical samples

A total of forty-two samples obtained from canine patients from different veterinary clinics of Santiago de Chile were processed. The first twenty samples were obtained from March to July 2010 in dogs with a marked symptomatology compatible with the neurological phase of DC, all were stored at 4ºC and then processed in August of 2010. The following samples were obtained from twenty-two dogs from different veterinary clinics from September 2010 to March 2011, with different symptomatic stages with suspected DC, which were kept at 4ºC. The inclusion criteria for these samples considered that all were positive for the specific ELISA test for CDV (positive IgM). In turn, they were processed in a shorter period than two weeks since obtaining it from the animals. In the table below shows the characteristics of the patients from whom the last twenty-two samples were extracted obtained (Table 1).

Identification of the samples positive to CDV by means of the detection of the H gene

All the samples were subjected to the test of the Polymerase Reaction in Chain associated with reverse transcription (RT-PCR) for the detection of the H gene of the CDV. Subsequently, the visualization of the amplified material was made by electrophoresis in 2% agarose gel. In the gel it was possible to visualize in three clinical samples (corresponding to the Charlotte, Facundo and Estrellita-2 patients) and in two vaccine strains a band corresponding to the DNA fragment of compatible size at expected: greater than 500 base pairs (Figure 1).

Sequencing and determination of the percentage of nucleotide identity of the samples positive for RT-PCR

Two of the positive samples were sent for sequencing to the company Genytec® (corresponding to the patients Facundo and Estrellita-2 (Table 1)). Once the sequences were obtained, their percentage of nucleotide identity was determined with respect to sequences published in the BLAST database. Within the first fifty aligned sequences, all correspond to CDV. In addition, both sequences yielded a percentage of nucleotide identity higher than 96% with respect to the first one hundred CDV sequences stored in Genbank®. This unquestionably demonstrates that the samples obtained correspond to CDV (Table 2 and 3).

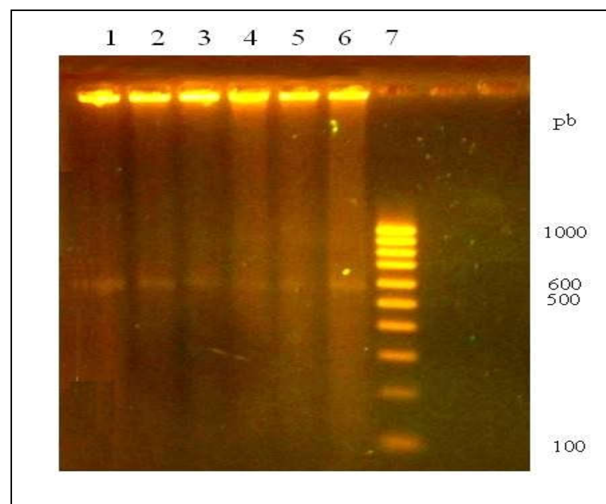

Figure 1. Visualization of products amplified by RT-PCR by electrophoresis in 2% agarose gel in TAE buffer and subsequent incubation in ethidium bromide. Lanes number 1, 2 and 3 correspond to clinical samples of patients Charlotte, Facundo and Estrellita-2 respectively (see table 1). Lane number 4 corresponds to the positive control vaccine Lederle strain. Lanes number 5 and 6 correspond to positive vaccine controls strain Onderstepoort. Lane number 7 corresponds to the molecular size marker with fragments between fifty and a thousand bp. The arrow indicates the display of DNA bands of a size close to six hundred bp

Table 1. Characteristics of peripheral blood samples from IgM positive dogs to DC. The race is defined, approximate age, whether or not they were immunized with the vaccine for distemper, the degree of neurological symptoms and the antibody titers obtained

Name	Race	Age	Vaccine?	Neurological signology	Ac IgM
Happy	Half blood	6M	no	+	1:80
Cholo	Half blood	4 Y	no	++	1:80
Josefa	Half blood	2 Y	no	++	1:80
Black Jack	Half blood	2,5 Y	yes	++	1:40
Charlotte	Half blood	1Y	no	++	1:80
Martin	Boxer	2Y	yes	++	1:40
Sofia	Half blood	8m	no	++	1:80
Snoopy	Beagle	1Y	yes	++	1:80
Lazy	Half blood	4Y	no	+	1:40
Rosita	Half blood	3Y	no	+	1:80
Estrellita	Half blood	2,5Y	yes	+++	1:80
Cachupin	Half blood	6Y	no	++	1:640
Pascual	Half blood	2Y	yes	+	1:80
NN	Half blood	2Y	?	+	1:80
Milo	Labrador	2Y	yes	+	1:40
Agustina	Half blood	2,5Y	no	+++	1:80
Renata	Half blood	3,5Y	no	+	1:80
Tontin	Half blood	2Y	?	+	1:40
Tatán	O. Aleman	8Y	?	+	1:40
Cookie	Half blood	2Y	no	++	1:20
Facundo	Boxer	2Y	no	++	1:40
Estrellita 2	Half blood	1Y	yes	+	1:80

Phylogenetic Analysis

From both samples sent three sequences were obtained, which were aligned using the Clustal Ω program, obtaining a consensus sequence for each sample: from the patient Facundo the sequence CDV13/RSV/Chile and the patient Estrellita-2 was obtainedCDV12/RSV/Chile. The phylogenetic tree of the CDV12/VSR/Chile sample was constructed using a region of fifty hundred and sixty-five base pairs together with different CDV sequences representing the eight officially recognized lines. The results of the alignment allowed knowing the corresponding geographic pattern of segregation; thus, the sequence CDV12/VSR/Chile segregated in the European lineage while the sequence CDV13/VSR/Chile did it in the lineage America 1 (Figure 2).

Table 2. Consensus sequence (Clustal Ω) and NIP (BLAST) from sample CDV/VSR12/Chile

Consensus sequence

ATGTATGAATTGTTGAAAGAGGATATGGAGAAATTAGAGGCTCGTACATTCACCAAGTCATAGATGTCT
TGACACCGCTCTTCAAAATTATTGGAGATGAGGTTGGGTTACGGTTGCCACAAAAACTAAACGAGATCA
AACAATTTATCCTTCAAAAGACAAACTTCTTCAATCCGAACAGGGAATTCGACTTCCGCGATCTCCACT
GGTGCATTAATCCACCTAGTAAGATCAAGGTGAATTTTACTAATTATTGCGATACAATTGGGATCAGAA
AATCTATTGCATCGGCAGCAAATCCCATCCTTTTATCAGCACTCTCAGGAGGCAGAGGTGACATATTCC
CACCATACAGATGCAGTGGAGCTACTACTTCAGTGGGCAGAGTTTTCCCCCTATCAGTATCATTGTCCA
TGTCTTTGATCTCAAGAACATCAGAGATAACCAA

	Max score	Total score	Query cover	E value	Ident	Accession
Canine distemper virus isolate CLdog14_06 hemagglutinin (H) gene, partial cds	784	784	98%	0.0	99%	KU052891.1
Canine distemper virus isolate CLdog14_05 hemagglutinin (H) gene, partial cds	784	784	98%	0.0	99%	KU052890.1
Canine distemper virus isolate CLdog15_03 hemagglutinin (H) gene, partial cds	784	784	98%	0.0	99%	KU052888.1
Canine distemper virus isolate CLdog14_17 hemagglutinin (H) gene, partial cds	778	778	98%	0.0	99%	KU052897.1
Canine distemper virus isolate CLdog14_15 hemagglutinin (H) gene, partial cds	778	778	98%	0.0	99%	KU052896.1
Canine distemper virus isolate CLdog14_04 hemagglutinin (H) gene, partial cds	778	778	98%	0.0	99%	KU052889.1
Canine distemper virus isolate lbmicdv25 hemagglutinin (H) gene, partial cds	778	778	98%	0.0	99%	KP202314.1

Table 3. Consensus sequence (Clustal Ω) and NIP (BLAST) for sample CDV/VSR13/Chile

Consensus sequence

AAAAGAAAAAGAAAGAAGAGTTATATAGTATTTAGTATTGGTGGGAATATGTCACCTCTGCCTCCTGAGA
GTGCTGATAAAAGGATGGGATTTGCTGCCGATGCAATGATTTTCTGATCCCAATTGTATCGCAATAATTA
GTAAAATTCACCTTGATCTTACTAGGTGGATTAATGCACCAGTGGAGACGCGGAAGTCGAATTCCCTGTT
CGGATTGAAGAAGTTTGTCTTTTGAAGGATAAATTGTTTGATCTCGTTTAGTTTTTGTGGCAACCGTAACC
CAACCTCATCTCCAATAATTTTGAAGAGCGGTGTCAAGACATCTATGACTTGGTGATGTACGGCCTCTGAT
TTCTCCATATCCTCTTTCAGCAATCTGCTAAATTCCATATTGCTAGTTGATACTTGGTGAAATCGAACTCC
AGTGATAGCAAGCAAGGTCATGATTCCAACCAGTAGGTGA

	Max score	Total score	Query cover	E value	Ident	Accession
Canine distemper virus isolate CLdog14_05 hemagglutinin (H) gene, partial cds	761	761	92%	0.0	99%	KU052890.1
Canine distemper virus isolate CLdog15_03 hemagglutinin (H) gene, partial cds	761	761	92%	0.0	99%	KU052888.1
Canine distemper virus isolate CLdog14_17 hemagglutinin (H) gene, partial cds	756	756	92%	0.0	98%	KU052897.1
Canine distemper virus isolate CLdog15_10 hemagglutinin (H) gene, partial cds	756	756	92%	0.0	98%	KU052893.1
Canine distemper virus isolate CLdog15_09 hemagglutinin (H) gene, partial cds	756	756	92%	0.0	98%	KU052892.1
Canine distemper virus isolate CLdog14_06 hemagglutinin (H) gene, partial cds	756	756	92%	0.0	98%	KU052891.1
Canine distemper virus strain Dog_GRE-7 hemagglutinin gene, complete cds	756	756	92%	0.0	98%	JN008899.1

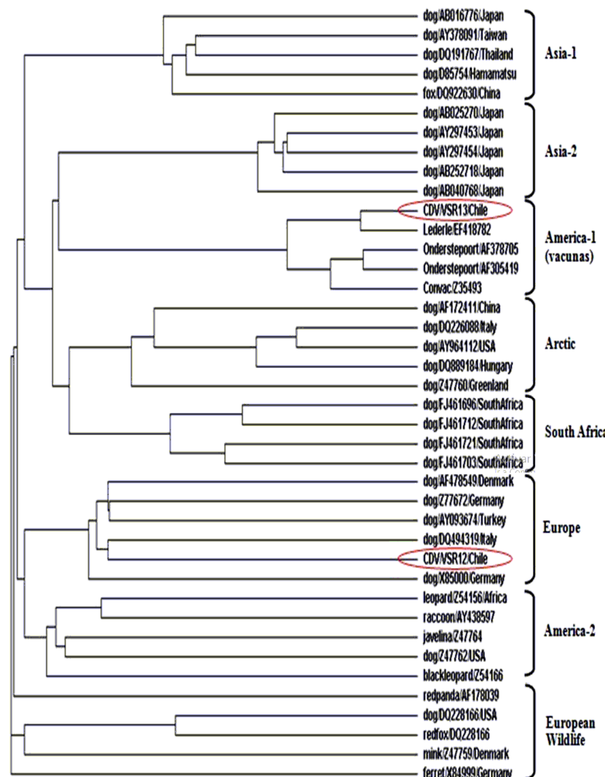

Figure 2. Phylogenetic relationship between strains of CDV taken from GenBank® with sample CDV/VSR12/Chile and CDV/VSR13/Chile, using the Clustal Ω program. Different strains of CDV representing each main lineage currently known were used. The species, access number for GenBank® and where it was isolated are indicated in each sample. The blue rectangle indicates the place where the sample CDV/VSR12/Chile and CDV/VSR13/Chile were associated, the European and América-1 lineage, respectively

DISCUSSION

For decades, the control of DC in domestic dogs in the world has been based mainly on vaccines prepared with modified live virus of the Americas 1 lineage (Ondersteeport, Leederle, etc). Currently, several countries describe an increase in the number of infected people despite being dogs with their vaccination program up to date (Martella *et al.,* 2008). In consideration of the above, possible hypotheses regarding this notorious and worrying increase in infection in domestic dogs, among which it is possible to highlight the circulation of new genetic variants of the virus, poor vaccination programs or a limited protection power acquired with conventional vaccines. According to the literature consulted, currently in Chile there are no epidemiological studies that allow to identify -if there are- genomic, antigenic or biological differences between different national isolates of the virus versus vaccine strains
Thus, the results of this work show that forty-two samples of peripheral blood from dogs were able to detect only in three a fragment of DNA of around five hundred pairs of bases and two of them were sent to sequence. Although it is known that the PCR method is a highly sensitive diagnostic tool, the question of possible false negatives is raised in the first twenty samples in the first instance for the time that they were stored in refrigeration from their collection to their processing in the laboratory, and that it is known the lability of viral RNA after its extraction, which can easily be degraded by the action of RNAases present in the skin and by temperatures above 4 ° C. Another hypothesis is the phase in which the disease was found in the patients, where the virus was possibly no longer found at the blood level but mainly in the epithelial tissue.

For the following samples, all were considered positive for the CDV-specific IgM test with antibody titers greater than 1:40 and worked within a period of less than two weeks from obtaining the host to processing. Of the twenty-two samples obtained in three of them, it was possible to detect a DNA fragment of the desired size corresponding to the Charlotte, Facundo and Estrellita-2 patients, in addition to two vaccine samples corresponding to the Leederle and Onderstepoort strains (Figure 1). Possible false negative hypotheses in the other samples is that the error can be human (incorrect instrument adjustment, sample contamination, diagnostic protocol not yet defined, etc.) due to the inexperience of working with the H gene as a diagnostic tool or due to the lability of the viral RNA.It is important to point out that the three positive samples presented high seroneutralization titers for distemper and in turn they were the ones that had less time from their extraction from the host to their processing in the laboratory. These characteristics are ideal to implement the PCR method in RNA viruses and confirms that it is possible to use primers for the H gene as a diagnostic tool for CDV (Jara, 2011). Currently, thanks to the sequence analysis of the H gene it has been feasible to classify the strains of CDV obtained within eight main lines or accepted genetic lineages worldwide (Martella *et al.,*2008) and those with the greatest genetic and antigenic diversity are the vaccine strains (lineage). America 1) with respect to the other CDV lineages (Beineke *et al*., 2009, Céspedes*et al*.,2010;Martella *et al.,*2008). By incorporating the sequences obtained and constructing the phylogenetic tree through the MEGA program, it was evidenced that the sequence CDV/VSR13/Chile corresponding to the patient Facundo segregates within the lineage America 1, the same group of vaccines (Figure 3). In this case the patient was not immunized against DC, therefore the detected virus corresponds to a field strain that circulates in the dog population. It could be inferred that the vaccines used in Chile could be effective for this group of viruses (lineage America 1) and that in this case the dog became ill due to not being vaccinated. However, to prove this claim, it would be necessary to experimentally challenge vaccinated dogs against national viruses of this lineage.On the other hand, the existence of the sequence CDV/VSR12/Chile from the patient Estrellita-2 would confirm that in Chile not only viruses of the lineage America 1 are present, but also viruses of the European lineage (Figure 2). On the other hand, the patient was vaccinated against DC, data that would support the hypothesis of new and different strains of the virus that would escape the protective power of conventional vaccines. However, this is not a totally guaranteed statement, since other relevant factors may exist, such as a bad vaccination program or the presence of maternal antibodies at the time of immunization, among others. In conclusion and under the results of this report, we can infer that an ideal vaccine should be designed considering the genetic polymorphism of the geographical area. Without However, in Chile there are no finished studies that allow the development of vaccine prototypes that consider the circulating genetic variants in the country (Céspedes*et al*.,2010). The importance of DC is not only restricted to domestic animal medicine; all over the world canine distemper disease is one of the main threats to the conservation of endangered wild species (McCarthy*et al.,*2007). Although there are no data to determine the situation of national wildlife, there are reports that recognize sporadic infection in fox's endemic to the forest of Fray Jorge (Moreira andStutzin,2005), there is a high possibility that urban dogs are acting as reservoirs of CDV in that area (Acosta-Jamett *et al.,*2011).

The risk to these populations requires the application of safe vaccines capable of generating a good immune response considering the national strains, since the presence of reservoirs of CDV can mean the sudden decline of a susceptible population, even reaching its extinction. Finally, this study has established the existence in Chile of two of the eight known lineages of CDV and that only one of them would be genetically related to the vaccine strains, which does not clarify the true epidemiological pattern that has taken the virus in the country, suggests the possible participation of other causal lineages of the disease, which could be related to the disturbing number of cases of dogs previously immunized and that acquire the disease. Although it is still a hypothesis that needs to be confirmed, it calls into question the need to update our national data regarding CDV, increase its epidemiological surveillance, have a good cellular and molecular characterization of the virus, identify if they are new national variants, and understand the epidemiological dynamics of the virus.

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