Genotypic evidence of infection by Canine Distemper Virus in maned wolf from a zoological collection in Chile.

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Abstract

Canine Distemper is one of the most important viral diseases described in domestic carnivores. However, with the passage of time, this disease has taken great relevance in wild animals. Thus, there are numerous international reports where several species such as wolves, foxes, ferrets, bears, seals and lions have been affected by this disease. In the month of April 2013, four maned wolves (*Chrysocyon brachyurus*) belonging to a zoo collection died presenting a clinical signology concordant with that produced by the distemper virus. Due to this, the need arises to carry out an accurate diagnosis to take the appropriate management measures and prevent other animals of the Zoo from being affected by this virus. For this, blood and organ samples were taken from the affected animals, which were sent to the laboratory of Virology and Microbiology of the Department of Animal Preventive Medicine of the Faculty of Veterinary and Animal Sciences of the University of Chile for its diagnosis. The purpose of this report is to determine by means of the Polymerase Chain Reaction associated with reverse transcription (RT-PCR), if the causal agent of the clinical illness presented by the maned wolves in the Zoo collection corresponds to the Canine Distemper Virus, and if so, to which of the existing lineages it belongs.

Keywords: CDV, lineages, maned wolves, captivity

INTRODUCTION

The canine distemper virus (CDV) is classified in the *Mononegavirales* order, *Paramyxoviridae* family and *Morbillivirus* genus. Antigenically, this virus has a close relationship with other morbilliviruses such as rinderpest virus and human measles virus (Carter *et al*., 2005; Pardo *et al.,* 2005). This pathogen is characterized by being a enveloped virus and helical nucleocapsid. It has a size between 150-300 nm and its genome is composed of unsegmented, single strand ribonucleic acid (RNA) and approximately 15.7 kb. It has a of negative coding sense and coding 6 viral proteins; Nucleocapsid protein (N gene), Phosphoprotein (P gene), Matrix protein (M gene), Haemagglutinin (H gene), Fusion protein (F gene) and Large polymerase protein (L gene). The haemagglutinin and the fusion protein, those responsible for inducing the production of neutralizing antibodies (Pardo *et al.,* 2005; Céspedes *et al.,* 2010). Hemagglutinin is the most variable protein described for all members of the genus *Morbillivirus*, it has been described that its amino acid sequence varies approximately 10% between the different strains of CDV (Martella *et al.,* 2008), a fact that could explain why CDV has a much wider host range than other viruses of the same genus (Nikolin *et al.,* 2012). Based on the analyzes performed on haemagglutinin, 14 circulating lineages of the CDV have been described, which are Africa -1; Africa-2; America-1; America-2; Asia-1; Asia-2; Asia-3; Asia-4; Europe-1 / South America-1; Europe-2 (wild European); Europe-3 (Arctic); Rockborn-like; South America-2; South America-3 (Panzera *et al.,* 2012; Sarute *et al.,* 2014; Bi *et al.,* 2015; Fischer *et al.,* 2016). Of the lineages present worldwide, studies carried out by Salas (2013), describe the presence of at least two of these in Chile; Europe-1 and America-1.

The illness. Being a virus that is not very stable in the environment, transmission occurs mainly through direct contact, through secretions or aerosols. CDV is eliminated by all secretions and excretions from the fifth day post-infection, before the clinical signs begin and continues even for weeks (MacLachland and Dubovi, 2011).

The infection usually occurs due to the invasion of the upper respiratory tract, followed by a massive replication of the virus in lymphoid tissue, producing a marked lymphopenia and immunosuppression (Nikolin *et al.,* 2012).

The incubation period can last from one to four weeks or even longer. During this period signs such as recurrent fever may appear, reaching its maximum at 3 days post-infection and is associated with the beginning of the spread of the virus to the organism. In addition, signs such as anorexia, mild depression, nasal and ocular discharge and tonsillitis can be observed. Systemic diffusion of the virus occurs ten days after infection and can be found in the epithelial cells of most organs (Céspedes *et al.,*2010).

Diagnosis. The great diversity of clinical signs makes the clinical diagnosis of the disease difficult and requires confirmation through laboratory tests. Due to this, there are several techniques that can be used, among these is the histological diagnosis, which reveals intra-cytoplasmic and intra-nuclear inclusion bodies in various tissues. In addition, there are serological methods, such as the immunoenzymatic (ELISA) and seroneutralization tests, which seek the detection of antibody (Ac) specific for CDV. The problem with these tests is that they are not capable of differentiating whether Ac (IgM against N and P proteins and IgG against H and F capside antigens) are of maternal origin, vaccines or product of infection (Nikolin *et al.*, 2012). Therefore, molecular techniques have been developed, such as the assay Polymerase Chain Reaction previous Reverse Transcription (RT-PCR) (Deem *et al.,* 2000; Martella *et al*., 2008; Pinotti *et al.,* 2009).

Epidemiology. The CDV has worldwide distribution and is the cause of a contagious and lethal multisystem disease in mammals belonging to the *Mustelidae* families (weasel, ferret, skunk, mink and otter); *Felidae* (cheetah, lion, jaguar, tiger, cat and ocelot); *Phocidae* (seals); *Procyonidae* (coati and raccoon) and *Ursidae* (bear), *Viverridae* (mongoose, cat osuno), *Hyaenidae* (hyena), *Ailuridae* (minor and giant pandas) and *Canidae* (dog, fox, wolf) (Carpenter *et al.,* 1998; Pardo *et al.,* 2005).

Within the Canidae family, there are several published cases of wild canids affected by CDV. As international examples, there are cases as described in Serengeti, Tazmania, where cases were reported in lions (Panthera leo), hyenas (*Crocuta Crocuta*) and foxes (*Otocyon megalotis*) (Carpenter *et al.,* 1998) and the case reported in Italy where red fox (Vulpes vulpes) were affected (Martella *et al.,* 2002). In the national case, there are those that occurred in 2003, in the Fray Jorge national park, where culprit foxes (*Pseudalopex culpeaus*) were affected by this virus (Moreira and Stutzin, 2005).

The maned wolf. The maned wolf (*Chrysocyon brachyurus*), also known as aguará guazú, guará wolf or boroche, corresponds to the largest canid in South America and was first described in the year 1801 by D'Azara (Fletchall *et al.,* 1995). Its distribution extends from central Brazil to northern Argentina, including eastern Bolivia, Paraguay and southeastern Peru (Ginsberg and MacDonald, 1990). It inhabits in flooded grasslands, bushes and wooded savannas (Rumiz and Sainz, 2002). Among its main physical characteristics, are its long legs, which are believed, would be an adaptation to see over the long pastures where it is often hunted (WAZA, 2013) and its large ears. The length of its body is 110-130 cm, the height at the

withers is 75-90 cm and it weighs around 23-35 kg (Fletchall *et al.,* 1995 and WAZA, 2013). Its fur is long and thick, it has a reddish-yellow mantle with darker hairs on its back. It has black legs and muzzle and the lower area is white as well as the tip of its tail (Ginsberg and Macdonald, 1990 and WAZA, 2013). It is an animal of twilight and nocturnal activity, it presents a solitary social system because it is a very territorial animal (Rumiz and Sainz, 2002; WAZA, 2013). It has an omnivorous diet (Rumiz and Sainz, 2002), being part of its diet small mammals, birds, fruits and insects (Ginsberg and McDonald, 1990). The World Conservation Union (IUCN) classifies it as Vulnerable, and since July 1979, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Fletchall *et al*. (1995), recommend for the immunization against CDV in maned wolves, a protocol that starts from 6 - 8 weeks with 3 doses, separated from 3 weeks, ideally, ending at 16 weeks. Then, at 6 months, revaccinate with a single dose and check the antibody titers existing at that time. It is said that titles about 1:30 are considered protective. At 12 months, a dose is once again administered, which is repeated once a year.

Considering the exposed background, in this work proposal will be analyzed blood samples from the Zoo collection,4manedwolvessuspectedofinfectionwithdistemper virus, in order to determine the presence of CDV in animals of the Zoo collection, by means the amplification of DNA fragment of approximately 560 base pairs, determine the percentage of nucleotide identity of the amplified fragment and know the CDV lineage present.

MATERIALS AND METHODS

Samples and controls. Three samples of viral RNA previously extracted from peripheral blood, collected in tubes with EDTA (2 ml) from maned wolves (*Chrysocyon brachyurus*), belonging to a Zoo collection, which presented clinical signs of the disease and which were immunized with the Recombitek vaccine. These samples are stored in the Virology and Microbiology laboratory at -20 ° C. As a positive control, RNA extracted from blood samples from dogs (*Canis familiaris*), positive for detection of the H gene by RT-PCR (Salas, 2013) will be used. As a negative control, samples of viral RNA extracted from blood samples with anticoagulants (EDTA), from dogs without clinical disease signs and that have not been vaccinated will be used. Finally, as a reagent control, sterile nuclease-free water will be used.

Detection of the CDV hemagglutinin gene by RT-PCR. Obtaining viral RNA: The extraction of the viral genome will be carried out using the TRIzol® LS kit (Invitrogen®), whose procedure is carried out at room temperature. 0.25 ml of sample will be mixed with 0.75 ml of reagent. Then, 0.2 ml of chloroform will be added to each tube, mixed for 15 seconds and then incubated at room temperature for 5 minutes. Subsequently, they will be centrifuged at 1200xg for 15 minutes and the aqueous phase will be transferred to a clean tube. For the precipitation of the RNA,0.5ml of isopropanol will be added, then incubated at room temperature for 10 minutes, and centrifuged at 12,000 xg for 8 minutes. The supernatant will be removed and washed 3 times with ethanol (1 ml of 75% ethanol), vortexed for 15 seconds and centrifuged at 7500xg for 5 minutes at room temperature. The supernatant will be removed, and the RNA pellet will be dried under vacuum for 10 minutes and resuspended in 0.1 ml of sterile, nuclease-free water. Finally, the RNA will be incubated at 55-60° C for 10 minutes and then kept at -20 ° C for use.

RT-PCR: For the implementation of the RT-PCR technique, an Apollo thermocycler (CLP, USA) of 96 wells of 0.2 ml each will be used and a protocol that involves temperatures and times for each stage, as well as the number of cycles required for the gene to be detected. Primers: The pair of primers to be used for the PCR reaction correspond to CDV1: 5'-GTCCTTCTCAT

CCTACTGG-3' and CDV2: 5'-ACACTCCGTCTGAGATAGC-3', which generate a DNA fragment of 561 base pairs (bp)) (Pardo *et al*., 2005).

Mixing of the reaction. The RT-PCR reaction will be carried out using the "SuperScriptTM one step RT-PCR with platinum Taq" kit (Invitrogen®) plus the CDV1 and CDV2 starters, following the manufacturer's instructions, which consists of 25 uL of "2x Reaction Mix" (0.4mM of each deoxyribonucleotide and 3.2 mM of MgSO4), 2uL of "SuperScript. III RT / Platinum Taq Mix ", 2 uL of each primer and 19 uL of RNA annealing to reach a total volume of 50 uL. The first step consists of the reverse transcription to be carried out at 60 ° C for 40 minutes and then an initial denaturation at 94 ° C for 2 minutes. Then a PCR sequence of 35 cycles (denaturation: 94ºC for one minute, alignment: 50ºC for 2 minutes, extension 72ºC for 2 minutes) and a final elongation at 72ºC for 2 minutes (Pardo *et al.,* 2005).

Visualization of the amplified products: It will be carried out by electrophoresis in 2% agarose gel in Tris acetate EDTA (TAE) buffer.The product of each PCR (5µL) will be mixed with the commercial loading product "6X Mass Ruler Loading Dye Solution" (1µL) (Fermentas®), to verify the progress of migration of DNA bands. As a molecular size marker Hyperladder I (Bioline®), which contains DNA fragments between 50 and 1000 bp, will be used. The electrophoresis will be performed at 90V for 90 minutes. After this, incubation of the gel with ethidium bromide (0.5 µg / mL) (Fermelo®) for 35 minutes will be performed and it will be visualized under ultraviolet light in a trans-illuminator (Transiluminator UVP®), to finally be photographed with digital camera.

Biosafety measures. All the time of laboratory work, safety measures will be implemented according to the biosafety levels established for the Microbiology and Animal Virology laboratory. This consists of the use of clean material, white long-sleeved apron and disposable latex gloves. In addition, an acrylic plate and goggles with UV protection will be used to protect from the ultraviolet light generated by the trans-illuminator used for the visualization of the amplified products. Finally, the gel submerged in ethidium bromide and the gloves used will be deposited in a container specially destined to be incinerated, because this compound has mutagenic properties.

Determination of the nucleotide identity of the amplified fragment. All samples that are positive for RT-PCR will be sequenced, which will be purified using the "HiYieldTM Gel / PCR DNA Fragments Extraction Kit" kit (RBC Bioscience®), according to the manufacturer's instructions. Then, these samples will be sent, in triplicate, to the Sequencing Center of the company Genytec Ltda. The sequences delivered by Genytec Ltda. will be aligned using the open access online program Clustal Ω, from this, a consensus sequence will be obtained for each sample, which will be entered the online access free access software BLAST to establish the nucleotide identity of the DNA fragments obtained in the RT-PCR, with respect to the existing sequences in GenBank®.

Analysis of results. Those samples that after the RT-PCR originate a fragment of DNA of approximately 560 bp and whose percentage of nucleotide identity, delivered by the BLAST analysis, show that the amplified fragment corresponds to the H gene of CDV will be considered positive.

Phylogenetic analysis. The phylogenetic analysis of the sequences obtained as positive will be carried out through the MEGA program of bioinformatic analysis.

RESULTS

Realization of the RT-PCR for the hemagglutinin gene. They were subjected to the Polymerase Chain Reaction test prior to Reverse Transcription for the detection of the CDV H gene; three positive controls, one negative control, four RNA field isolates from maned wolves and reagent control. Later, for visualization, positive controls were loaded into the agarose gel, generating a high intensity band of around 500 bp, negative control and reagent control. Of the four field isolates, two did not generate bands, being considered negative to the detection of the H gene, and two generated visible bands of high intensity and approximately 500 bp, being considered positive to the detection of the CDV H gene (Figure 1).

Figure 1
Visualization of the products amplified by RT-PCR in 2%agarose gel electrophoresis incubated in ethidium bromide. Lanes number 1 and 2 correspond to the RNA samples of the wolf 4517. Lanes number 3 and 4 correspond to RNA samples from wolf 7490. Lanes numbers 5, 6 and 7 correspond to the positive controls. Lane number 8 corresponds to the negative control. Lane number 9 corresponds to the molecular size marker (50-1000 bp) and finally, lane number 10 to the reagent control.

Sequencing and determination of the percentage of nucleotide identity of the amplified fragments. Through the nucleotide alignment performed for both positive samples (Annex 1), two consensus sequences were obtained: "Chile/lobocrin/1"for wolf 4517and "Chile / lobocrin/2" for wolf 7490 (Table 1).

Chile/lobocrin/1

TGTTGTGTTTTTTTTCCCTTCCTCACCTACTGGTTGGAATCATGACCTTGCTTGCTATCACTGGAGTT
CGATTTCACCAAGTATCAACTAGCAATATGGAATTTAGCAGATTGCTGAAAGAGGATATGGAGAAATC
AGAGGCCGTACATCACCAAGTCATAGATGTCTTGACACCGCTCTTCAAAATTATTGGAGATGAGGTTG
GGTTACGGTTGCCACAAAAACTAAACGAGATCAAACAATTTATCCTTCAAAAGACAAACTTCTTCAAT
CCGAACAGGGAATTCGACTTCCGCGATCTCCACTGGTGCATTAATCCACCTAGTAAGATCAAGGTGAA
TTTTACTAATTATTGCGATACAATTGGGATCAGAAAATCTATTGCATCGGCAGCAAATCCCATCCTTT
TATCAGCACTCTCAGGAGGCAGAGGTGACATATTCCCACCAATACTAAATACTATATAACTCTTCTTT
CTTTTTCTTTTTTTTTCCCCTCCCCCCCCCCCGCCCTCCCTTTCCTCGCCCGCGCCTCTCTC

Chile/lobocrin/2

AGTGTTGTGTCCTTTTTTCCCCTACTGGTTGGATACATGACCTTGCTTGCTATCACTGGAGTTCGATT
TCACCAAGTATCAACTAGCAATATGGAATTTAGCAGATTGCTGAAAGAGGATATGGAGAAATCAGAGG
CCGTACATCACCAAGTCATAGATGTCTTGACACCGCTCTTCAAAATTATTGGAGATGAGGTTGGGTTA
CGGTTGCCACAAAAACTAAACGAGATCAAACAATTTATCCTTCAAAAGACAAACTTCTTCAATCCGAA
CAGGGAATTCGACTTCCGCGATCTCCACTGGTGCATTAATCCACCTAGTAAGATCAAGGTGAATTTTA
CTAATTATTGCGATACAATTGGGATCAGAAAATCTATTGCATCGGCAGCAAATCCCATCCTTTTATCA
GCACTCTCAGGAGGCAGAGGTGACATATTCCCACCAATACTAAATACTATATAACTCTTCTTTCTTTT
TCTTTTCCTTCCCCCTCCCCCACCCCCTCCCTCCCTTTCCTCGCCCGCGCCTTTCTCGCAGAT

Table 1:
Consensus sequences obtained for the two samples amplified and aligned by Clustal Ω program

Both consensus sequences were entered the BLAST program, to identify the origin of the amplified DNA fragments in the RT-PCR. The first fifty sequences aligned correspond to CDV, in addition, due to the high percentage of nucleotide identity obtained (99-98%) with respect to the sequences stored in GenBank®, it is possible to corroborate that both amplified fragments correspond to CDV.

Phylogenetic analysis.

The analysis was carried out using 90 nucleotide sequences of the H gene region: 81 sequences of isolates from different geographical areas, six sequences of the Phocine Distemper Virus (PDV), two sequences of the isolates obtained in this title memory and one sequence of the Virus of measles as an external group. According to the tree constructed, both samples (Chile/lobocrin/1 and Chile/lobocrin/2) segregate in the lineage Europa-1 (Figure 2).

DISCUSSION

The canine distemper is one of the most important viral diseases described in domestic carnivores. However, its relevance is not only restricted to pet medicine, since some years ago there were outbreaks of the disease throughout the world and a significant expansion in the range of hosts (Appel and Summers, 1999), making canine distemper in one of the main threats to the health and conservation of wild species (McCarthy *et al.,* 2007).

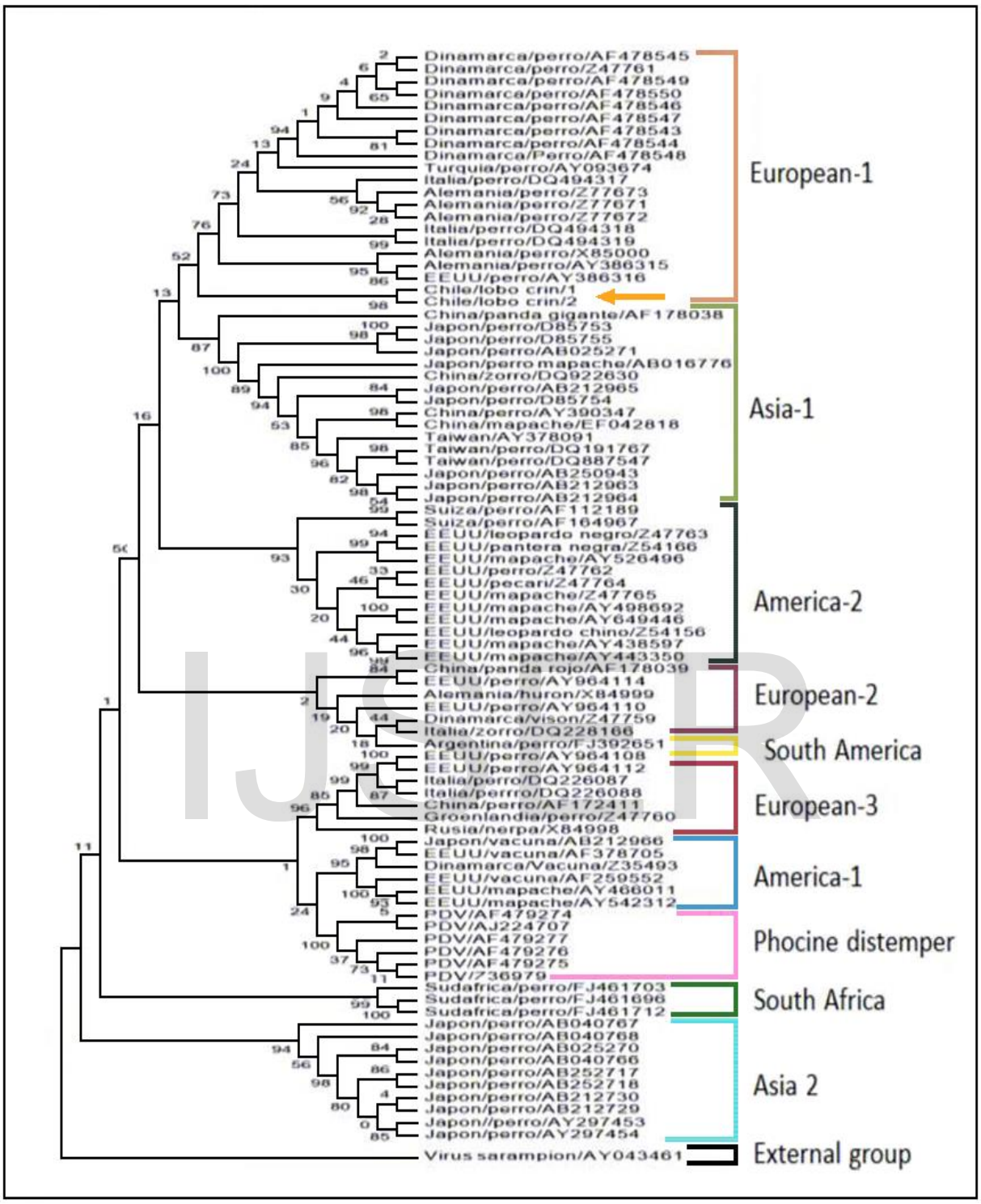

Figure 2

Evolutionary analysis carried out by the Mega 6 Program. The evolutionary distances were calculated by Tamura's 3-parameter method. The robustness of the tree was determined by means of a "boostrap" of 500 replicas. In total, 91 nucleotide sequences were included; the samples analyzed in this report are highlighted with a yellow arrow.

According to the literature consulted, at a national level there are no data that determine the CDV's situation in wildlife, either free-living or in captivity. There is a report in which it is described that the populations of culpeo foxes (*Pseudalopex culpeaus*) and chillas (*Pseudalopex griseus*) belonging to the Fray Jorge National Park (IV Region), apparently were affected by this disease (Moreira and Stutzin, 2005). However, this antecedent is only based on samples from a few dead animals that were analyzed serologically and histopathologically and does not have direct diagnostic methods to confirm the causal infectious agent.

Subsequent studies indicate that the main reservoir for this geographical area corresponds to domestic dogs of urban areas, so it is suspected that the origin of the outbreak was due to dogs from the areas surrounding the Park (Acosta-Jammet *et al.*, 2011). Considering the foregoing, it is that the work done in this title report constitutes the first study conducted in Chile where CDV is confirmed as a cause of death in wild carnivores, through molecular characterization based on partial analysis of the sequence of the H gene of the CDV of field isolations from captive animals.

The RT-PCR technique used in this work, amplified a fragment of approximately 560 bp in the three positive controls and did not amplify for the negative control. The results of this investigation show that of four field samples analyzed, only two samples of peripheral blood were able to amplify a DNA fragment of approximately 500 bp. Although the RT-PCR technique is characterized by having a high specificity and sensitivity, the existence of possible false negatives in the other samples is considered. This may be due to the lability of the viral RNA, since can be easily degraded by the action of RNA-loops present in the skin and by temperatures above 4 ° C. Storage is also a factor to consider, since the RNA isolates used were stored at -20 ° C and described that viral RNA can remain indefinitely active at -70 ° C, - 192 ° C (liquid nitrogen) or lyophilized (*Pérez et al.,* 1993).

The phylogenetic analysis revealed that both consensus samples obtained segregate towards the Europa-1 lineage. Of the lineages described worldwide, recent studies by Panzera *et al.* (2015) describe that in South America, the most prevalent correspond to America-1 (vaccine strains) and the Europe-1 lineage, which would have entered the continent possibly by Brazil around 1975. The exception to this occurs in Argentina, where the SouthAmerica-2 lineage, unique to this country, is even more prevalent than the urope-1 lineage (Panzera *et al.*, 2015). At the national level, the only antecedent found about the lineages that circulate within the country, is the work done by Salas (2013), where analyzing blood samples from domestic dogs, he obtained that at least two of the world-described lineages are circulating in the Chilean canine population: Europe-1 and América.

Regarding the maned wolves, in the literature there are authors who describe that the CDV is one of the main causes of infectious diseases that affect these animals in captivity (Borges and Guimarães, 2001), however, this antecedent is only based in serological studies performed on different populations of these animals and does not have direct diagnostic methods.

Therefore, this work corresponds to the first confirmed report of natural infection in wolves of the mane in which the CDV can generate a clinical disease with a high mortality, and that its diagnosis is confirmed by molecular techniques.

In addition, the results obtained correspond to the first Latin American report of an epidemic outbreak by CDV in wild carnivore species, in which confirmation is made by molecular diagnosis.

Finally, the results obtained show that the CDV corresponds to a threat to wild populations in captivity at the national level, since it can generate disease (with high mortality) in susceptible species present in them. In addition, these results generate new questions about the possible origin of the outbreak, opening the door to new research to obtain information on the national state of the CDV in wildlife in captivity.

Conflict of interest: The Author(s) declare(s) that there is no conflict of interest.

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