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Promising use of Polymerase Chain Reaction Associated to Reverse Transcription for the Detection ofthe America-1 Lineage of Canine Distemper Virus

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Abstract: *Canine Distemper (CD) is one of the pathologies that causes the highest rate of morbidity and mortality in domestic canines worldwide and also represents an important disease in several families of terrestrial mammals: Canidae, Procyonidae, Mustelidae, Mephitidae, Hyaenidae, Ursidae ,Ailuridae, Viverridae, Felidae and some marine mammals the cape seal (pusacaspica). This disease is caused by Canine Distemper virus (CDV), a single-stranded RNA virus and negative polarity, whose genome consists of six genes, codes for six structural proteins. Among them, the Hemagglutinin encoded by the H gene, has the highest amino acid variability, induces the production of neutralizing antibodies synthesized by the host immune system. Based on the variability of the H gene, it has been described that worldwide there would be at least fourteen lineages different from the CDV and in our country has been described the presence of at least two lineages circulating among our sick dogs with the pathology: America-1 and Europe.*

Thus, the objective of this report was to implement the detection of the America-1 lineage of the Canine Distemper Virus by means of the Polymerase Chain Reaction associated with reverse transcription (RT-PCR) by means of in silico-designed primers. For this, the access numbers of the described nucleotide sequences were used in a phylogenetic tree constructed using the CDV H gene as target (Ke et al., 2015) and stored in the Genbank® database,

The designed primers were found to be effective for CDV detection and the RT-PCR was able to detect a specific fragment of the H gene from the positive samples, therefore it could be suggested that these in silico-designed primers can effectively be used to detect the America-1 lineage.

Keywords: *DC, CDV, America-1 lineage, Gen H, primers, in silico, RT-PCR.*

1. INTRODUCTION

Canine Distemper (CD) is one of the main diseases that affects domestic canines and causes high morbidity and mortality rates in unvaccinated dogs. In recent years it has been observed in the clinic that animals vaccinated in the same way are catching the virus. Canine Distemper Virus (CDV) also affects common and endangered species of wild animals throughout the world. Domestic dogs and other related species serve as reservoirs of the virus and play an important role in the transmission of the disease (Chinnadurai *et al*., 2017). CDV can affect many species, the younger ones being more susceptible, because they have lost maternal antibodies and their immune system is still immature to respond to infection (Martella *et al*., 2008). The genome of the virus encodes six structural proteins: nucleocapsid protein encoded by the N gene, membrane protein encoded by the M gene, phosphoprotein encoded by the P gene, large polymerase encoded by the L gene, hemagglutinin encoded by the H gene and the fusion protein encoded by the F gene (Martella, *et al*., 2008). CDV lineages have been described based on the analysis of the Hemagglutinin (H) gene, this gene has the highest degree of variability. At present, 14 types of lineages have been determined according to this gene, identified as America-1 (vaccine strain), America-2, Asia-1, Asia-2, Asia-3 and Asia-4, Europe1/South America-1 (EU-1 / SA-1), Arctic, European Wild (EW), South America-2 (SA-2), South America-3 (SA-3), Rockborn-like (RL), Africa-1 and Africa -2 (Ke *et al*., 2015). In Chile, it was determined through an analysis of a region of the H gene, that at least two circulating lineages would exist in the country's canine population: America 1 and Europe 1 (Salas et al., 2018). Because DC is a highly contagious disease, lethal and affecting many species, it is very important to implement a technique that allows antemortem viral detection and thus facilitate diagnosis and early treatment. Thus, in this report specific *in silico* primers were designed for the América-1 lineage of

the CDV H gene, using the Polymerase Chain Reaction (PCR), prior reverse transcription. The expected result would allow positioning the H gene as a detection target despite the variability described.

2. BACKGROUNDS

2.1. Ethiology

The Canine Distemper Virus (CDV) is classified in the *Mononegavirale*s order, *Paramyxoviridae* family and *Morbillivirus* genus (Amarasinghe *et al*., 2018). It is a pleomorphic virus, has a lipid envelope and a diameter of 150 to 300 nm. (Summers and Appel, 1994). Its genome consists of about 15.7 kilobases (kb), consisting of a single strand of RNA of negative polarity, not segmented, which codes for six structural proteins: nucleocapsid protein (N gene, 1.5 kb), the membrane protein (M gene, 1 kb), two proteins associated with transcriptase: the phosphoprotein (P gene, 1.5 kb), the large polymerase (L gene, 6.5 kb), two types of glycoproteins; hemagglutinin (gene H, 1.8 kb) and the fusion protein (gene F, 1.9 kb) (Martella, *et al*., 2008; Céspedes *et al*., 2010). Proteins F and H, located in the lipid envelope, induce the production of neutralizing antibodies synthesized by the host's immune system (Appel and Summers, 1995). The helical nucleocapsid contains the N, P and L proteins, which initiate intracellular replication. The M protein connects the glycoproteins of the surface and the nucleocapsid during viral maturation (Beineke *et al*., 2009). The large polymerase protein (L) is the fundamental subunit of the polymerase RNA complex given its catalytic effect in the synthesis of viral RNA. (Lamb and Parks, 2007).

2.2. Host Range of the Canine Distemper Virus

The CDV hosts include numerous families of the order of carnivores such as Canidae (dogs, foxes, wolves among others), Procyonidae (raccoons, coati), Mustelidae (ferret, weasel, mink, among others), Mephitidae (skunks), Hyaenidae (hyenas), Ursidae (bears), Ailuridae (red pandas), Viverridae (genets, civets) and Felidae (tigers, lions, leopards, jaguars, with the exception of the domestic cat) (Martella et al., 2008; Chinnadurai et al., 2017. Yipeng, et al., 2017). In addition, it has been detected in some marine mammals such as the cáspica seal (pusa caspica) (Kuiken et al., 2006).

2.3. Pathogenesis

The main routes of entry of the virus are ocular, nasal and oral, through secretions, aerosols and fomites, reaching mucosal surfaces (von Messling et al., 2005). CDV is a lymphotropic and highly immunosuppressive virus (Pinotti et al., 2009). Rapid replication begins in local lymph nodes and in seven days to all lymphatic tissues (primary viremia), producing early infection of lymphocytes and mononuclear cells, by blocking the synthesis and signaling pathways of interferons and cytokines, decreasing the proliferation of B and T lymphocytes, the latter being more affected, resulting in severe immunosuppression of the host (von Messling et al., 2005). During the second and third week after infection, some dogs initiate a strong humoral and cellular immune response and can recover without clinical signs later, while others develop a weak immune response and present acute or subacute disease, because the lymphocytes and mononuclear cells infected carry the virus to the epithelial surface of the digestive, respiratory, urogenital, skin and / or central nervous system, with the respective clinical signs (secondary viremia) (Appel and Summers, 1999). In the Central Nervous System (CNS) the virus replicates initially in neurons and in glial cells, it can cause lesions in the white matter and in the gray matter. In a chronic course of infection, due to a deficient immune response or a delayed response, characteristic demyelinating inflammatory lesions are described (BSAVA, 2013).

2.4. Clinical Signs

CD has different clinical presentations that can vary from acute, subacute to chronic, where the respiratory, digestive and nervous systems are affected (Martella, et al., 2008). The severity of the clinical signs observed depends on different factors such as the virulence of the infecting virus, environmental conditions, the age of the host and their immunological status (BSAVA, 2013).

Systemic signs include anorexia, dehydration, fever and oculo-nasal serous discharge. Neurological signs include myoclonus, ataxia, tremor, seizures, visual deficit and vestibular dysfunction.

Neurological signs may be caused directly by the virus or may occur as a result of the immune response against the CNS, in which case the animals have a level of intermediate immunity and CNS involvement may develop months or years later (Gamiz et al., 2011; BSAVA, 2013).

2.5. Treatment

Due to the lack of specific antivirals and standardized protocols, the treatment used consists of a symptomatic therapy, support care (hydroelectrolytic fluids, vitamin and nutritional supplements) and antibiotics to prevent secondary bacterial infections, common in immunocompromised animals (Appel and Summers, 1999; Martella et al., 2008, Pinotti et al., 2009).

2.6. Prevention and Control

Vaccination is the main strategy to prevent and control the disease. Vaccines with live attenuated virus (VVA) stimulate the humoral and cellular immune response and induce immunological memory (Martella et al., 2008). The development and use of these vaccines have contributed to a drastic reduction in the incidence of DC in domestic dogs (Appel and Summers, 1999). Despite this, outbreaks of the disease have been observed in populations of immunized dogs belonging to different geographic regions (BSAVA, 2013). These outbreaks could be explained by the reversal of the virulence of the attenuated strains, by the emergence of new strains sufficiently variable to evade the immune response generated by the vaccines, failures in the administration of the vaccines, or, due to the immunological state of the animal (Salas et al., 2018).

2.7. Diagnosis

The diagnosis of DC is based on the clinical suspicion supported by the manifestation of clinical signs and the antecedents of risk predisposing to the disease (Martella et al., 2008). However, the lack of specificity in the symptoms associated with the infection can lead to confusion with other pathologies in the final diagnosis, for which various complementary diagnostic methodologies have been developed, including serological techniques (immunohistochemistry and ELISA). and molecular techniques (Polymerase Chain Reaction prior reverse transcription (RT-PCR) (Pinotti et al., 2009).

2.8. PCR and the RT-PCR Variant

The PCR technique was developed in the eighties and since then it has revolutionized molecular genetics, making possible the study of a wide range of genes. It has been used in multiple areas where the immense potential of PCR to amplify minimal amounts of DNA (or RNA) has been particularly attractive (Murphy et al., 1999). Recently it has been applied successfully, since it is a highly specific, rapid and sensitive method for the antemortem diagnosis of CDV infection, independently of the presentation of the disease, the humoral immune response, and the distribution of the viral antigen (Frisk et al., 1999; Pardo et al., 2005; Navarro, 2012). Thus, to apply this technique from RNA, it is necessary to do a reverse transcription before starting the amplification by PCR, this will generate a complementary DNA (cDNA), which will be the substrate for PCR (Elia et al., 2006).

2.9. CDV Lineages

Through RT-PCR, the existence of at least 14 CDV lineages based on the nucleotide sequence of the H gene has been established worldwide (Asia 1, Asia 2, Asia 3, Asia 4, America 1, America 2, Arctic, Europe 1/South America 1, South America 2, South America 3, Wild European, Africa 1, Africa 2 and Rockborn-like) (Ke et al., 2015).

In Chile, in a study it was established by means of the analysis of a segment of the H gene that there would be at least two circulating lineages in the national canine population, indicating the lineages America-1 and Europa-1 as present in the country, where America-1 is genetically related to vaccinal strains (Salas et al., 2018)

As already established that in the country there are at least two types of lineages circulating among sick dogs with CD and one of those lineages is also used in vaccines, in this work the use of RT-PCR was proposed. primers designed in silico- for the detection of CDV using the lineage América-1 of the H gene as target.

Thus, with the success of this strategy, the presence of the virus can be established and directly determine if the sample analyzed belongs to this lineage, suggesting the discarding of nucleotide sequencing.

3. Materials And Methods

The present study was carried out in the Microbiology and Virology Laboratories of the Department of Preventive Medicine of the Faculty of Veterinary and Animal Sciences (FAVET) of the University of Chile.

3.1. Design in Silico Primers for the Detection of the CDV Lineage América-1

First, an official database of genetic sequences (Genbank®, 2018) was used to obtain the nucleotide sequences of the Latin-1 lineage of the CDV, as described in previous phylogenetic tree (Ke et al., 2015). Then, Clustal Ω software (2018), free access, was used to align sequences to determine nucleotide identity zones, thus obtaining the candidate sequences to be used for the design of primers. This design contemplates the use of free access software (OligoPerfect ™ Designer, 2018). When there is more than one pair of candidate primers, the one that provided the best selection parameters was chosen, such as the percentage of GC and Tm. The specificity of the primers was corroborated by the BLAST program. Once the sequence of the primers was obtained, their synthesis was commissioned to Fermelo® and the lyophilized primers were solubilized to a final concentration of 1 uM.

3.2. Implement the RT-PCR Reaction Detection of the CDV America-1 Lineage

The obtained primers were used in an RT-PCR reaction that contemplated the use of 20 samples of RNA positive to CDV (N gene) found in the laboratory, obtained from the blood of dogs with CDV compatible signology. The "SuperScript ™ one step RT-PCR with PlatinumTaq" kit (Invitrogen®) was used according to the manufacturer's instructions. Following the protocol proposed by the company, which consisted of 25 uL of the component "2x Reaction Mix", which contains 0.4 mM of each deoxyribonucleotide and 3.2 mM of MgSO4, 2uL of "SuperScript. III RT / Platinum Taq Mix ", 5 uL of each primer and 5 uL of RNA annealing to reach a final volume of 50uL.

RT-PCR protocol: a 96-well Apollo 96-well thermocycler was used and the retrotranscription stage of the RNA was favored in order to obtain the complementary DNA to be used in the next stage carried out according to the manufacturer's instructions: 45 ° C for 30 minutes and then 94° C for 2 minutes. The DNA denaturation phase was carried out at 94° C for 30 seconds. During the alignment phase a temperature of 53° C was used for 30 seconds and the elongation phase contemplated a temperature of 72° C for one minute. After 40 cycles, we proceeded to a final extension stage at 72° C for 8 minutes and then, we continued with the visualization of the amplified product.

Two samples typified as América-1 and European lineage was used as a positive an negative control, respectively (Salas et al., 2018 (Salas, 2013) and nuclease-free water was used as reagent control.

3.3. Visualization of the DNA Fragment Synthesized in RT-PCR

The products were visualized by means electrophoresis in 2% agarose gel in Tris-HC1 buffer (100 mM Tris-HC1, 10 mM EDTA) and their path compared to a molecular size standard (AccuRuler 100bp Plus DNA Ladder, Maestrogen®). 5 µL of the PCR product was taken and mixed with 1 µL of commercial loading product (Fermentas®). Next, 90 V electrophoresis was performed for ninety minutes. After electrophoresis the gel was incubated with ethidium bromide (0.5 µg / mL) for thirty minutes, the DNA bands were visualized in an ultraviolet light transilluminator. Biosecurity measures. Use of clean material, disposal of waste and use of closed white apron and gloves during the development of practical work. Now of visualizing the gels, glasses with UV filter were used, and after use, the gels incubated in ethidium bromide were eliminated through the use of incineration, since the chemical has mutagenic properties (Saeidnia and Abdollahi, 2013).

4. Results

Design in silico primers for the detection of the America-1 CDV lineage.

4.1. Obtaining Nucleotide Sequences

The nucleotide sequences were obtained from the official database of genetic sequences (Genbank®, 2018), occupying the access numbers of the 11 nucleotide sequences (Table 1).

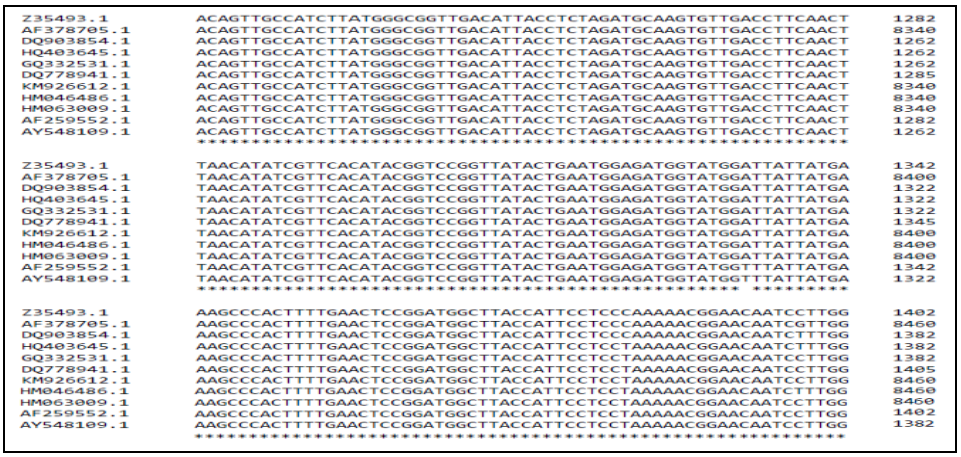
Table1. *Genbak® Access Numbers used to Obtain Common Nucleotide Zones for the America-1 Lineage*

Viral strain	Access number	Isolation year	Geographic location
America-1			
AF378705_30_ZA_Onderstepoort_Fox	AF378705	1930s	USA
Z35493_40_US_Convac_Dog	Z35493	1930-1950	USA
AF259552_40_US_SnyderHill_Dog	AF259552	1930-1950	USA
DQ903854_51_US_Lederle_Dog	DQ903854	1951	USA
AY548109_98_US_2655_Raccoon	AY548109	1998	USA
HM046486_07_KZ_Phoca_Seal	HM046486	2007	Kazakstan
HM063009_89_KZ_Shuskiy_Mink	HM063009	1989	Kazakstan
KM926612_92_CN_L_Fitchew	KM926612	1992	China
DQ778941_06_CN_CDV3_Mink	DQ778941	2006	China
GQ332531_08_CN_16_Dog	GQ332531	2008	China
HQ403645_09_CN_GZ1_Dog	HQ403645	2009	China

4.2. Alignment of Sequences

The Clustal Ω software was used for the alignment of sequences and the nucleotide identity zones were determined. In this way, we obtained the candidate sequences to be used for the design of primers as shown in Table 2, where the alignment of the eleven nucleotide sequences is demonstrated, and in the areas where there are zones of nucleotide identity it is shown with an asterisk at the end of each column.

Table2. *Example of the use of the Clustal Program Ω and of the alignment of common candidate sequences to obtain primers.*



```
Z35493.1        ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  1282
AF378705.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  8340
DQ903854.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  1262
HQ403645.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  1262
GQ332531.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  1262
DQ778941.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  1285
KM926612.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  8340
HM046486.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  8340
HM063009.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  8340
AF259552.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  1282
AY548109.1      ACAGTTGCCATCTTATGGGCGGTTGACATTACCTCTAGATGCAAGTGTTGACCTTCAACT  1262
                ************************************************************

Z35493.1        TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGATTATTATGA  1342
AF378705.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGATTATTATGA  8400
DQ903854.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGATTATTATGA  1322
HQ403645.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGATTATTATGA  1322
GQ332531.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGATTATTATGA  1322
DQ778941.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGATTATTATGA  1345
KM926612.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGATTATTATGA  8400
HM046486.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGATTATTATGA  8400
HM063009.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGATTATTATGA  8400
AF259552.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGTTTATTATGA  1342
AY548109.1      TAACATATCGTTCACATACGGTCCGGTTATACTGAATGGAGATGGTATGGTTTATTATGA  1322
                ************************************************ *********

Z35493.1        AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCCAAAAACGGAACAATCCTTGG  1402
AF378705.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCCAAAAACGGAACAATCGTTGG  8460
DQ903854.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCCAAAAACGGAACAATCTTTGG  1382
HQ403645.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCTAAAAACGGAACAATCTTTGG  1382
GQ332531.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCTAAAAACGGAACAATCCTTGG  1382
DQ778941.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCTAAAAACGGAACAATCCTTGG  1405
KM926612.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCTAAAAACGGAACAATCCTTGG  8460
HM046486.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCTAAAAACGGAACAATCCTTGG  8460
HM063009.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCTAAAAACGGAACAATCCTTGG  8460
AF259552.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCTAAAAACGGAACAATCCTTGG  1402
AY548109.1      AAGCCCACTTTTGAACTCCGGATGGCTTACCATTCCTCCTAAAAACGGAACAATCCTTGG  1382
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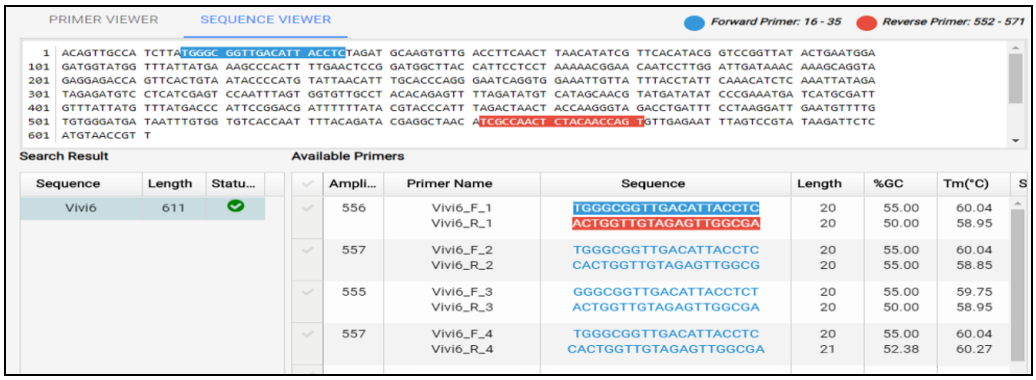
4.3. Primers Design

There were several segments of the genome with areas with nucleotide identity zones, however, the primers that could be obtained from these sequences had a low percentage of guanine-cytosine, generated smaller amplicons, the fusion temperature was very variable among the partisans and some of those primers could be united to several lineages of the CDV. For this reason, the zone indicated in Table 3 was chosen, where the one that provided the best selection parameters, such as the percentage of GC and Tm, was chosen. The primers would allow obtaining an amplicon of about 556 base pairs (bp).

Table3. *Use of the OligoPerfect Design® Program by Invitrogen for the in vitro design of CDV primers*



PRIMER VIEWER | SEQUENCE VIEWER — Forward Primer: 16 - 35 — Reverse Primer: 552 - 571

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  1 ACAGTTGCCA TCTTATGGGC GGTTGACATT ACCTCTAGAT GCAAGTGTTG ACCTTCAACT TAACATATCG TTCACATACG GTCCGGTTAT ACTGAATGGA
101 GATGGTATGG TTTATTATGA AAGCCCACTT TTGAACTCCG GATGGCTTAC CATTCCTCCT AAAAACGGAA CAATCCTTGG ATTGATAAAC AAAGCAGGTA
201 GAGGAGACCA GTTCACTGTA ATACCCCATG TATTAACATT TGCACCCAGG GAATCAGGTG GAAATTGTTA TTTACCTATT CAAACATCTC AAATTATAGA
301 TAGAGATGTC CTCATCGAGT CCAATTTAGT GGTGTTGCCT ACACAGAGTT TTAGATATGT CATAGCAACG TATGATATAT CCCGAAATGA TCATGCGATT
401 GTTTATTATG TTTATGACCC ATTCCGGACG ATTTTTTATA CGTACCCATT TAGACTAACT ACCAAGGGTA GACCTGATTT CCTAAGGATT GAATGTTTTG
501 TGTGGGATGA TAATTTGTGG TGTCACCAAT TTTACAGATA CGAGGCTAAC ATCGCCAACT CTACAACCAG TGTTGAGAAT TTAGTCCGTA TAAGATTCTC
601 ATGTAACCGT T
```

Search Result

Sequence	Length	Statu...
Vivi6	611	✓

Available Primers

Ampli...	Primer Name	Sequence	Length	%GC	Tm(°C)	S
556	Vivi6_F_1	TGGGCGGTTGACATTACCTC	20	55.00	60.04	
	Vivi6_R_1	ACTGGTTGTAGAGTTGGCGA	20	50.00	58.95	
557	Vivi6_F_2	TGGGCGGTTGACATTACCTC	20	55.00	60.04	
	Vivi6_R_2	CACTGGTTGTAGAGTTGGCG	20	55.00	58.85	
555	Vivi6_F_3	GGGCGGTTGACATTACCTCT	20	55.00	59.75	
	Vivi6_R_3	ACTGGTTGTAGAGTTGGCGA	20	50.00	58.95	
557	Vivi6_F_4	TGGGCGGTTGACATTACCTC	20	55.00	60.04	
	Vivi6_R_4	CACTGGTTGTAGAGTTGGCGA	21	52.38	60.27	

4.4. Primer Specificity

The specificity of the primers was corroborated by the BLAST program. Where it was indicated that both primers can be joined to CDV sequences. Once the sequence of the primers was obtained and the BLAST was made to each of the primers, Fermelo® was commissioned for its synthesis. Objective 2 Implement the RT-PCR reaction for the detection of the América-1 lineage of the CDV. With the procedure indicated in Material and Method, unique and clear DNA bands between 500 and 600 bp were visualized for the positive control (CDV lineage America-1) and in the 20 positive samples to CDV according to the N gene. No bands were observed in the lanes corresponding to the negative control (CDV European lineage) or in the control of reagents (Figure 1).

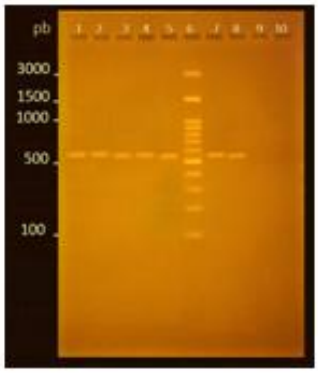

Fig1. *Gel electrophoresis 2% agarose*

Lane 1: Control (+) lineage America 1; Lane 2: sample 1; Lane 3: sample 5; Lane 4: sample 8; Lane 5: sample 10; Lane 6: MTM; Lane 7: sample 14; Lane 8: sample 17; Lane 9: control (-) European lineage; Lane 10: reagent control; MTM: Maestrogen® (100-3000 bp)

To corroborate the negative sample (European lineage) and to eliminate the possibility of degradation of the RNA involved, an RT-PCR was additionally performed to detect the N gene according to the protocol previously established in this laboratory (Muñoz, 2013). Clear and unique DNA bands of a size close to 300 bp are observed and no nonspecific bands are observed (Figure 2).

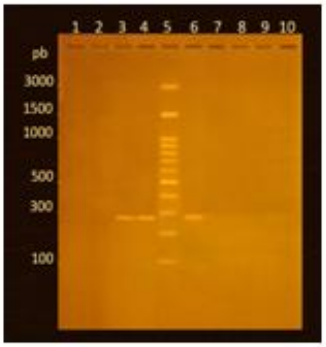

Fig2. *Gel electrophoresis Agarose 2%.*

Lane 1: Control (-); Lane 2: control of reagents; Lane 3: control (+) European lineage.

Lane 4: control (+) lineage America. Lane 5: MTM; Lane 6: sample 19; Lanes 7-10: without loading. MTM: Maestrogen® (100-3000 bp).

5. DISCUSSION

The H gene is one of the six genes that make up the CDV genome and codes for the glycoprotein Hemagglutinin, which in addition to generating the immune response in the body (Appel and Summers, 1995), has the highest antigenic and genetic variability, presenting around of 10% variability between different lineages. Therefore, several authors describe it as a gene not suitable for the molecular diagnosis of the virus (Gallo *et al.*, 2007, Martella *et al.*, 2008).

In the Faculty of Veterinary and Animal Sciences of the University of Chile, two reports were made that used the H gene as a detection target in the RT-PCR technique (Jara *et al.*, 2018, Salas *et al.*,

2018). In the first, five out of six stored samples with a week old and none of the 20 oldest samples stored were amplified. In the second, three of the 42 samples analyzed by its protocol were amplified. In both cases, the sensitivity of their RT-PCR protocols was low, from which it could be inferred that these results were obtained due to the primers used and described in previous studies (Mochizuki *et al*., 1999; Pardo, 2006).

In this work, an RT-PCR protocol with primers designed in silico was used, using the H gene as a detection target and a fragment of about 556 bp was amplified in the 20 samples positive to CDV according to the N gene. Negative samples were the existence of degraded RNA samples; however, negative samples were corroborated as intact RNA samples by applying an RT-PCR with primers for the N gene (Figure 2).

According to these results, the protocol used in this Title Memory could be used in samples suspicious to CDV, to be able to define if this protocol has high sensitivity and specificity to detect CDV lineage América-1. The specificity of busy primers could also be corroborated using other programs such as Vector NTI or DS gene. However, to corroborate the sensitivity of the method implemented, additional studies are required.

It is recommended to obtain the nucleotide sequences of the DNA fragments obtained from the samples, to compare them with CDV isolates available in GenBank®, in this way it will be possible to confirm that the samples do indeed belong to the América-1 lineage.

6. CONCLUSION

The molecular test implemented represents a rapid and specific ante-mortem diagnostic method for Canine Distemper disease, which is effective for the detection of CDV. The RT-PCR was able to detect a specific fragment of the CDV H gene from the positive samples, therefore it could be suggested that these in silico designed primers can effectively be occupied to detect the America-1 lineage

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