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Is the M gene of Canine Distemper virus a eligible target for detection?

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Abstract: The Canine Distemper as one of the main infectious diseases that affect both domestic and wild canines as well as several other mammals both terrestrial and marine and the difficulty of its diagnosis based on clinical signs, has motivated several studies have been carried out in the Faculty of Veterinary and Animal Sciences of the University of Chile to detect the etiological agent: The Canine Distemper Virus. The molecular technique used is the Polymerase Chain Reaction associated with reverse transcription and the different genes that make up its genome have been used as a detection target: H, P, F, L, N find a method optimal that allows the diagnosis of ante-morten disease early. Thus, in this report, when using the M gene, which codes for the protein of the Virus Matrix, a molecular detection protocol was implemented that completes the information regarding which would be the optimal gene to be used in the diagnosis of the disease. For this, 20 samples of total RNA were used, obtained from peripheral blood of dogs of different breeds, ages and vaccination status against CDV, which showed clinical signs of DC and which were already positive to the RT-PCR that detects the N gene of canine distemper virus. The technique detected the viral genome in 70% of those analyzed, generating a band close to 500 bp, whose amplified products presented clear intensity and sharpness to the visualization. The nucleotide identity percentage (NIP) with respect to the two consensus sequences obtained from the total samples (using the Clustal Omega online program) yielded 99% for both sequences compared to the Canine Distemper Virus sequences delivered by free access online BLAST software. These results allow to conclude that although the choice of the M gene as a detection target has been successful, it is not of choice for an accurate detection of the canine distemper Virus.

Keywords: Canine Distemper, M gene, Virus Matrix

INTRODUCTION

The virus

The canine distemper virus (CDV) belongs to the order *Mononegavirales*, family *Paramyxoviridae*, genus *Morbillivirus*, which is taxonomically related to other *Morbillivirus* of this genus as the Human Measles Virus (VS), Bovine Virus, Pest Virus of Small Ruminants (Rinderpest), Seal Distemper Virus and Dolphin Distemper Virus (Pringue, 1999, Lamb and Kolakofsky, 2001, Martella *et al*., 2008). It is the causal agent of Canine Distemper disease (CD), which is one of the main infectious pathologies of young and adult dogs, being considered the most serious and important viral pathology that affects the species, both wild and domesticated canines (Appel, 1987). Its viral etiology was demonstrated by the French scientist Henri Carré, in 1905, who demonstrated that the infection could be transmitted with a filterable agent. Before that date, in 1809, Edward Jenner had described the virus (Pardo *et al*., 2005, Martella *et al*., 2008, Pinotti *et al*., 2009, Pratelli, 2011).

CDV is a enveloped virus, with a size between 150 and 300 nm in diameter, whose genome is composed of ribonucleic acid (RNA), unsegmented, single-stranded and negative polar, consisting of 15.7 kb in length or 15,700 nucleotides, with a nucleocapsid of helical symmetry (Örvell, 1980, Diallo, 1990, Sidhu *et al*., 1993, Summers and Appel, 1994, Elia *et al*., 2006). It presents six genes, whose function is to code the six structural proteins of the virion, these genes are N, P, M, F, H, L, where each of them encodes a single protein, being the exception the P gene that encodes the structural protein P (phosphoprotein) and non-structural proteins V and C (Lamb and Parks, 2007). Genes or gene regions are organized into separate and non-overlapping transcriptional units, which are organized from 3'-N-P-M-F-H-L-5 ', (Lamb and Kolakofsky, 1996, Harder and Osterhaus, 1997, Barrett, 1999).

The structural proteins are: nucleocapsid protein, encoded by the N gene (1.5 kb);

phosphoprotein, encoded by the P gene (1.5 kb); polymerase major, encoded by the L gene (6.5 kb); matrix protein, encoded by the M gene (1 kb) and, two surface glycoproteins of the lipid envelope, fusion protein, encoded by the F gene (1.9 kb), and hemagglutinin, encoded by the H gene (1,8 kb), both responsible for the recognition and entry of the virus into the host cell, constituting the main objectives of the antibodies generated by it (Sidhu *et al*., 1993, Summers and Appel, 1994, Shin *et al*., 2004) .

The virus remains viable between pH values of 4.5 to 9, is sensitive to ultraviolet light, to heat, to desiccation and is destroyed when exposed to temperatures of 50 to 60 ° C for 30 minutes; however, it can survive for one hour at 37 ° C. The survival time is prolonged in cold temperatures, being viable between 0 and 4 ° C for 2 or 3 weeks. Stored at -65°C can be stored for more than seven years. Being a enveloped virus, it is sensitive to ether, chloroform and disinfectants such as formaldehyde (0.5%), phenol (0.75%) and quaternary ammoniums (0.3%), making routine disinfection procedures they are effective in destroying it (Appel, 1977). The extracted viral RNA can be very labile, being able to be degraded easily by RNAsas of the skin and by temperatures higher than 4 ° C. The CDV stored at -10 ° C remains active only for about a month and in freezers at -70 ° C or -192 ° C (liquid nitrogen) or lyophilized, it can remain active indefinitely (Pérez *et al*., 1993).

The illness.

It corresponds to a multisystem viral disease, worldwide distribution, contagious and in many cases fatal, being most terrestrial carnivores, the most susceptible to natural infection by CDV (Appel and Summers, 1995). The families of mammals that affect the virus besides the family Canidae (dogs, foxes, wolves, coyotes and jackals), and that are also susceptible to the natural infection by this, are diverse terrestrial carnivores as members of the *Mustelidae* family (otters, ferrets, mink and badger), *Procyonidae* (coati, raccoon and lesser panda) (Appel and Summers, 1999), *Felidae* (wild felids such as lions, tigers, leopards in captivity) (Appel *et Al.*, 1994), *Hyaenida* (hyenas), *Ursidae* (bears, giant panda), *Viverridae* (civeta and genere), *Ailuridae* (red panda) (Appel and Summers, 1995), in which the virus has been detected. It also affects marine carnivores such as seals, which besides having their specific distemper virus, can become infected with the CDV, and cetaceans like the dolphin (Appel and Summers, 1999, Pardo *et al.*, 2005, Beineke *et al.*, 2009). CDV is considered highly contagious and classified as the cause of diseases of great economic concern, conservation and public health (Harder and Osterhaus, 1997, Deem *et al.*, 2000). It can also compromise drastically the conservation of threatened species due to its high lethality (Pardo *et al.*, 2005). Therefore, it continues to be one of the main problems for veterinarians and pet owners due to the high morbidity and mortality rates (Patel *et al*., 2012). The release of the virus occurs mostly through the oro-nasal route, although it can be found in any discharge and secretion, and its main routes of entry to the body are ocular, respiratory and oral, through direct contact through the inhalation of the CDV transported by air or droplets (Krakowka *et al.*, 1980), reaching mucosal surfaces where the first interaction with the host's immune system is established by early infection of local lymphocytes and mononuclear cells (von Messling *et al.*, 2005; Parks, 2007), which is important since the virus is not stable in the environment and is rapidly inactivated in the environment (Greene and Appel, 1998, Elia *et al.*, 2006), hence the inhalation of secretory aerosols. Respiratory viruses, is the most important route of transmission of canine distemper (Appel and Summers, 1995). After it enters, it affects mucous membranes and lymphoid tissues, replicating mainly in the lymphatic tissues of the respiratory tract where macrophages and monocytes located in tonsils and respiratory epithelium are the first type of cell to replicate and propagate viruses (Appel, 1970). After this local replication, the first phase of viremia occurs leading to a generalized infection of all lymphoid tissues including spleen, thymus, lymph nodes, bone marrow, mucosal-associated lymphoid tissues (MALT) and macrophages in the lamina propria of the gastrointestinal tract, as well as Kupffer cells; This wide proliferation in lymphoid organs corresponds to the initial increase in body temperature and fever develops and lymphopenia and leukopenia begins, mainly caused by viral damage to lymphoid cells (Appel, 1969, Appel, 1970, Krakowka *et al.*, 1985). Days later, the second viremia occurs, which leads to the infection of cells of parenchymal tissues throughout the organism (Appel, 1969, Okita *et al.*, 1997) and in dogs that do not recover early, infected lymphocytes and macrophages transport the virus. virus to the surface of the epithelia of the digestive, respiratory, urogenital tracts and the Central Nervous System (CNS) (Appel and Summers, 1999). The elimination of the virus begins before the clinical signs appear, from the fifth day post infection, and continues to be eliminated even for weeks (Maclachland and Dubovi, 2011). Dogs with acute infection begin to eliminate the virus by all body secretions and can be excreted 60 to 90 days after exposure, (Greene and Appel, 1998, Pardo, 2006). The diversity in symptoms, associated with the unpredictable and variable cycle of the virus in the host, makes it difficult to establish a precise ante-mortem clinical diagnosis in most affected animals (Frisk *et al.*, 1999). The clinical symptoms of Distemper are similar to those of other pathologies, frequently causing confusion in the clinical diagnosis (Demeter et al., 2007). In those cases where an animal, especially a puppy, presents febrile symptoms and multisystemic

symptoms, it should be considered as one of DC prediagnostics (Martella *et al.,* 2008).

As a first sign, in general, one can find lethargy, dehydration, anorexia and weight loss, with marked clinical manifestation depending predominantly on the affected organ, and in the case of fever, this is a biphasic fever (Wright *et al.,* 1974).

In the acute phase, the clinical signs that can be found are mild skin rashes (rashes), serous discharge or nasal and ocular mucosa, conjunctivitis and anorexia, followed by gastrointestinal and respiratory signs, which are often complicated by secondary bacterial infections and neurological problems. (Krakowka *et al.,* 1985). Nervous signs are diverse and progressive and include myoclonus, nystagmus, ataxia, postural deficit and tetraparesis or paralysis (Greene and Appel, 1998, Koutinas *et al.,* 2002, Vandevelde and Zurbriggen, 2005, Amude *et al.,* 2007).

The disease in domestic dogs is a disease that affects dogs of all ages, being the puppies of 3 to 6 months of age the most susceptible because they have lost the maternal antibodies and their immune system is still immature to respond against the infection (Martella *et al*., 2008).

In our country, the presence of the CDV was empirically suggested in 1994, which was the year in which the first isolation of this virus is produced and reported from dog kidney cell cultures (MDCK, Madin-Darby Canine Kidney) inoculated with nasal secretions, eye and tracheal obtained from a canine that showed clinical signs of DC; was a puppy with respiratory dyspnea, bronchial rales and nervous signs, with unilateral myoclonus, involuntary masticatory movements and ascending paresis of the posterior train. The diagnosis was corroborated by electron microscopy and histopathological studies that demonstrated the presence of acidophilic intracytoplasmic inclusion bodies (Cerda *et al.,* 1994). Subsequently, an isolate of a viral strain diagnosed as CDV was reported by immunofluorescence (Navarro *et al.,* 2002). In this case, the sample had been obtained from nervous tissue of an adult dog with nervous symptoms.

The diagnosis

The diagnosis of CDV infection is complicated by the great variety of clinical signs as well as by the few reliable tests to detect the disease in the initial stages, and its signs may be confused with those of other diseases, since the main clinical signs are present, including nasal discharge and diarrhea, are common to other respiratory and enteric diseases (Choi, 1990, Jones et al., 1997). However, although multisystemic disease is easy to recognize, this is not always the case, and there are even non-classical neurological manifestations that make diagnosis difficult. Despite the above, one should always have the Canine Distemper within the prediagnostics, especially in the case of a puppy that presents febrile conditions and multisystemic symptoms (Martella, et al., 2008). Therefore, the confirmation of the diagnosis is by laboratory tests that allow the exclusion of other diseases with similar clinical manifestations (Elia et al., 2006, Pardo, 2006, Maclachland and Dubovi, 2011). Among the most used diagnostic techniques are histology, which investigates the presence of intranuclear and intracytoplasmic inclusion bodies in numerous tissues. Serology is also used as an indirect diagnostic study, through the enzyme-linked immunosorbent assay (ELISA, Enzyme-Linked Immuno Sorbent Assay) and seroneutralization assays that detect specific antibodies for CDV, and molecular techniques such as the RT- PCR assay (Elia *et al.,* 2006; Pardo, 2006; Martella *et al.,* 2008; Sarute *et al.,* 2014). Histology investigates presence of intranuclear and intracytoplasmic inclusion bodies, however, these can only be visualized at certain periods of the disease, they are generally absent when clinical signs are generated (Summers and Appel, 1994, Martella *et al.,* 2008).

In the case of ELISA, it is aimed at the detection of specific IgM against CDV, which persists in dogs infected with the virus from five weeks to three months, depending on the strain and host response; In vaccinated dogs, IgM persists for approximately 3 weeks (Bernard *et al.,* 1982). False negatives can be observed in dogs that die acutely without the presence of an immune response and can also occur in subacute or chronic presentations. High IgM titers are specific to diagnose recent CDV infections, however, recent vaccination with modified live virus may give false positive results. The ELISA assay has been shown to have high specificity and sensitivity compared to the virus neutralization test (Waner *et al.,* 2006). The disadvantage of serological tests lies in not being able to differentiate between maternal, vaccine or infection (Frisk et al., 1999) although the tests are reliable, the problem occurs when interpreting the results, constituting a disadvantage for false positives that occur if post immunization is performed with a vaccine (Elia et al., 2006). In the cerebrospinal fluid (CSF), the serology aims to find an increase in anti-CDV immunoglobulin (IgG and IgM) titers suggestive of CDV infection, and if an increase in protein concentration is observed (> 25 mg / dL) and cell count (10 cells / mL with a predominance of lymphocytes) would be against inflammatory signs of CDV, as in encephalomyelitis (Elia et al., 2006; Pardo, 2006). This is pathognomonic in dogs with an intact blood-brain barrier, but its absence does not exclude CDV infection (Appel and Summers, 1999). Another diagnostic method of DC is the isolation of the virus, but this is a very slow method to perform (Elia et al., 2006), which

may take about 3 weeks. It is possible to isolate CDV in the laboratory, isolation that presents a very high sensitivity and specificity, however, it is a laborious and slow process so it is not done routinely (Reutemann *et al.*, 2006; Martella *et al.*, 2008).

The Polymerase Chain Reaction associated with reverse transcription.

The Polymerase Chain Reaction associated with reverse transcription (RT-PCR), consists of an exponential amplification of DNA fragments of the virus, previous reverse transcription from RNA to complementary DNA, allowing to detect the presence of the virus RNA early, being, unlike the other mentioned techniques, a sensitive, specific and rapid test for the diagnosis of CDV, being positive even when other tests fail to detect the virus (Appel and Summers, 1999; Frisk et al., 1999; Martella *et al.* , 2008). In the last decades, several methods based on RT-PCR for the diagnosis of CDV have been developed, being the main targets for amplification, genomic regions that present a high degree of conservation among virus isolates. Thus, like Frisk et al., 1999, they developed a method based on the amplification by RT-PCR of a conserved fragment of 287 bp of the N gene of the CDV, which was later used by several authors, proving to be a very effective technique for the detection of the virus genome (Frisk *et al.*, 1999; Gallo Calderón *et al.*, 2007).

At the Faculty of Veterinary and Animal Sciences of the University of Chile, in the search to find the best method to detect CDV, title memories using RT-PCR have been carried out for this purpose, using as target the different genes of the virus where, for example, Jara (2011) and Salas (2013), focused on the gene of hemagglutinin (gene H). Another RT-PCR was also developed based on the detection of the nucleocapsid protein gene (N gene) by Muñoz (2013). Finally, Mateo (2015) focuses on the viral phosphoprotein gene (P gene) as target and Pincheira (2015) on the large polymerase gene (L). The results of Pincheira's work (2015) were 100% positive, unlike those who used the H gene as target, where their results yielded positive values of 7% (Salas, 2013) and 83% (Jara, 2011), and in the case in which the test was used in the N gene, its result was 91% (Muñoz, 2013) of positive results in the detection of CDV.

Considering what has already been mentioned and, apparently, there are no works that use the M gene of the CDV as target, in this work a molecular diagnostic method was established by the RT-PCR technique using the M gene of the CDV as a target for detection, a highly conserved gene, 1 kb, responsible for coding the protein of the matrix (M), a structural protein of the virion constituted by 335 amino acids, being. This protein, positioned under the envelope, has a fundamental role in the organization of the virus morphogenesis and in the assembly of the virion, due to the interactions it establishes with the integral membrane proteins, the lipid bilayer and the nucleocapsid (Sidhu et al.., 1993, Summers and Appel, 1994, Lamb and Parks, 2007).

The results obtained in the present work provide another antecedent to the information that is already available regarding the detection of the virus by this molecular technique, RT-PCR, using as a target the genes that make up the genome and thus be able to elucidate in together with previous works, which of the methods performed results in a better technique that allows obtaining a more accurate and effective diagnosis of disease and thus adopt the necessary measures to avoid infection.

MATERIALS AND METHODS

The present work was developed in the Microbiology and Animal Virology laboratories, belonging to the Department of Animal Preventive Medicine of the Faculty of Veterinary and Livestock Sciences of the University of Chile. Samples The samples used for the RT-PCR reaction consisted of two strains of CDV from commercial vaccines (Lederle and Onderstepoort) as positive controls. Twenty samples of total RNA, obtained from peripheral blood of dogs of different breeds, ages and vaccination status against CDV, that showed clinical signs of DC and that were positive to the RT-PCR that detects the N gene. Two negative controls were used samples of total RNA extracted from blood samples from dogs without DC signology and negative to RT-PCR (N gene). For the control of reagents, nuclease-free water was used.

Primers design

We considered 4 known sequences of the M gene of CDV stored in the Genbank (Annex 1) whose size was greater than 1000 bases because it is expected to obtain a fragment of around 500 base pairs to be sent to be sequenced. The mentioned sequences were aligned according to the free Clustal Omega online access program (Annex 2) and the consensus sequence (Annex 3) was obtained and entered into the online free access program OligoPerfectTM Designer from LifeTechnologiesTM, which provided both the sequences of the primers used that generated a DNA fragment of around 500 bp, as well as the melting temperature (Tm) that served as the basis for the determination of the alignment temperature by means of a temperature gradient thermocycler (Annex 4). The designed starters: (P1: CAATAACACCCCATTGCACA and P2: ATCTCCA TGAGCGGGTAACA) were synthesized by IDT (Fermelo, Chile).

Implementation of the RT-PCR technique

A 96-well 96-well Apollo thermal cycler (CLP, USA) was used. The RT-PCR protocol contemplated the indication of the commercial kit supplier (Super Script one step RT-PCR with platinum Taq, from Invitrogen®): initial incubation at 50 ° C for 40 minutes and then at 94 ° C for 2 minutes, followed by 30 cycles of PCR (denaturation at 94 ° C for 45 seconds, alignment at 55 ° C for 45 seconds and extension at 72 ° C for 45 seconds). The reaction mixture contemplated 15 µL of the designated commercial kit (which includes the enzymes RT and Taq DNA polymerase, MgCl2 and the deoxyrubonucleotidestrifostatoses), 5 µL of sample and 5 µL of each specific splitter (called P1 and P2 respectively), reaching a final volume of 30 µL. Detection of amplified products. A 2% agarose gel (Winkler®) gel electrophoresis was performed in Tris acetate EDTA (TAE) buffer (Fermentas®). 5 µL of the PCR product was taken and mixed with 1 µL of a commercially available 6X Mass Ruler Loading Dye Solution (Fermentas®) product containing bromophenol blue to observe the progress in migration of the DNA bands. The electrophoresis was carried out at 90V for 90 minutes. After electrophoresis, the gel was incubated in ethidium bromide (0.5 µg / mL) (Sigma®) for 35 minutes. The bands were then visualized in a transilluminator of ultraviolet (UV) light (Transiluminator UVP®). As a molecular size marker, Maestrogen® was used, which contains DNA fragments between 100 and 3000 bp, with which the size of the amplified fragments was compared. Finally, the observed result was photographed with a digital camera and a suitable filter.

Determination of the nucleotide identity of the amplified fragment

It was performed by sequencing 2 positive samples to RT-PCR, which were purified through the kit "HiYield Gel / PCR DNA Fragments Extraction Kit" (RBC Bioscience®) and sent in triplicate to the Sequencing Center of the company Genytec Ltd. the nucleotide identity percentage (NIP) of the sequences delivered by Genytec Ltd. was obtained after they were aligned with the purpose of obtaining a consensus sequence through the online free access program Clustal Ω, which was entered into the computer program of BLAST free access online sequence alignment, in order to know its nucleotide identity with respect to the first 15 results delivered by GenBank®. For the analysis of the results, a positive sample was considered as the one that originated a fragment of DNA of approximately 500 bp and with a PIN greater than 90% with respect to the result delivered by the BLAST program (official sequences of the M gene).

Biosecurity measures consisted in the use of long-sleeved and closed apron, latex gloves for the handling of potentially harmful or toxic products, use of acrylic plate as a protective filter when the ultraviolet light transilluminator was used, and the gels used , they were wrapped in latex gloves, deposited in a specially designed container, and then incinerated, while the material contaminated with virus was sterilized in the autoclave.

RESULTS

Detect the M gene of the Canine Distemper Virus using primers designed *in silico.*

Using the designed starters (Annex 4), an RT-PCR was performed with the 20 positive CDV RNA samples (according to the N gene) and the respective controls. The visualization of the 2% agarose gel showed the presence of unique and clear fluorescent bands of around 500 bp. No presence of fluorescent bands was observed in the negative controls or in the control of reagents, obtaining positivity in 14 of the 20 samples analyzed (70%). (Figure 1)

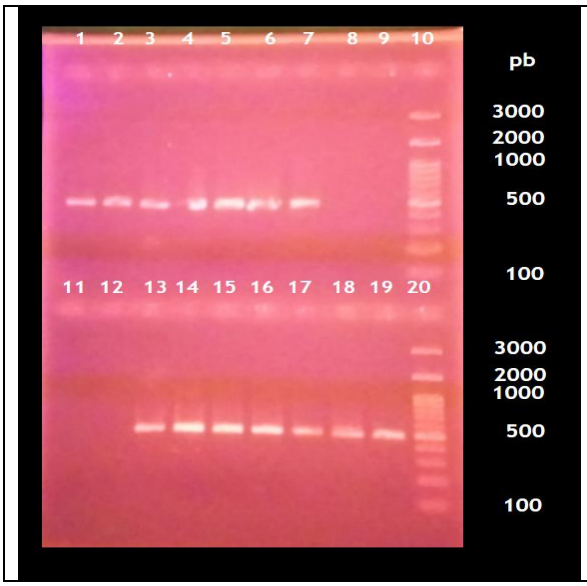

Figure 1: 2% agarose gel electrophoresis of RT-PCR of RNA samples from N-positive CDV and the respective controls.

Lanes 1-5: positive samples; lanes 6 and 7: vaccine controls; lane 8: negative control; lane 9: reagent control; lanes 11-19: positive samples; lanes 10 and 20: MTM: Maestrogen® (100-3000 bp).

Establish the nucleotide identity of the amplified DNA fragment

Due to the cost involved, only two of the 20 samples amplified to CDV (N gene) amplified were sent to be sequenced (in triplicate). The sequences received from Genytec Limited are shown in Annex 5 and 6. Using the Clustal Ω online program (Annex 5 and 6), a consensus sequence was obtained for each trio of sequences: MG1C and MG2C.

>MG1C

ACACCCCATTGCACATCTTAACTCCGTGGAAGAAGGTCCTTACGAGTGGGAGTGTGTTCAATGCAAATCAA GTCTGTAACGCAGTCAATTTAATACCATTAGACATAGCACAGAGATTTAGGGTAGTATACATGAGCATCAC TCGACTATCAGACGATGGAAGTTACAGAATTCCTCGCGGGATGTTTGAATTCCGCTCCAGGAATGCTTTAG CATTTAATATTTTAGTCACCATTCAAGTTGAGGGAGATGTCTGTTCAAGCCGAGGTAATTTGAGCATGTTC AAAGATCACCAAGTGACATTCTTGGTGCATATCGGCAACTTTAGCCGGAAGAAGAACCAAGCTTACTCTGC TGATTACTGTAAACTGAAAATTGAAAAGATGGGATTAGTGTTTGCTCTAGGAGGGATAGGGGGAACCAGT CTTCACATACGATGTACTGGTAAGATGAGCAAGGCTCTGAATGCCCAGCTAGGGTTCAAGAAAATCC

>MG2C

CCCATTGCACATCTTAACTCCGTGGAAGAAGGTCCTTACGAGTGGGAGTGTGTTCAGTTCAAATCAAGTCT GTAACGCAGTCAATTTAATACCATTAGACATAGCACAGAGATTTAGGGTAGTATACATGAGCATCACTCGA CTATCAGACGATGGAAGTTACAGAATTCCTCGCGGGATATTTGAATTCCGCTCCAGGAATGCTTTAGCATT TAATATTTTAGTCACCATTCAAGTTGAGGGAGATGTCTGTTCAAGCCGAGGTAATTTGAGCATGTTCAAAG ATCACCAAGTGACATTCATGGTGCATATCGGCAACTTTAGCCGGAAGAAGAACCAAGCTTACTCTGCTGAT TACTGTAAACTGAAAATTGAAAAGATGGGATTAGTGTTTGCTCTAGGAGGGATAGGGGGAACCAGTCTTC ACATACGATGTACTGGTAAGATGAGCAAGGCTCTGAATGCCCAGCTAGGGTTCAAGAAAACCCT

Tables 1 and 2 (Annexes 7 and 8) indicate a nucleotide identity percentage (NIP) of around 99% at CDV for both consensus sequences, when entered into the BLAST online program.

DISCUSSION

CD is a world-wide, contagious and fatal disease that mainly affects carnivores (Appel and Summers, 1999), where not only domestic animals are affected, but also constitutes one of the main threats to the conservation of endangered wild species. of extinction (Macarthy *et al.*, 2007); as in Chile with the fox chilote (*Pseudalopex fulvipes*), which can be inexorably threatened by the rapid infectivity and lethality characteristic of disease (Céspedes *et al*., 2010). It is produced by the CDV and given the diversity in the symptoms, associated with the unpredictable and variable cycle of the virus in the host, makes it difficult to establish a precise ante-mortem clinical diagnosis in most affected animals (Frisk *et al.*, 1999) and if one also takes into account that the clinical symptoms of DC are similar to those of other pathologies, it can frequently lead to confusion in the clinical diagnosis (Demeter *et al.*, 2007). The polymerase chain reaction (PCR) preceded by retrotranscription (RT) of genomic RNA is a molecular methodology that allows a rapid ante-mortem diagnosis, with a high degree of sensitivity and specificity (Frisk et al., 1999; Kim; et al., 2001; Gebara *et al.*, 2004; Saito *et al.*, 2006). Considering that the CDV genome has 6 genes, establishing which of them turns out to be the best target for detection by RT-PCR is interesting, since diagnostic studies prefer genes or genomic regions presenting as a target for amplification. high degree of conservation among isolates, such as the N, P and M genes (Frisk *et al*., 1999, Kim et al., 2001, Goller *et al.*, 2010, Si *et al.*, 2010), as opposed to H gene that presents high variability and would not therefore be the best choice as a diagnostic method (Jara, 2011) but rather, to know the lineage to which a circulating virus would belong (Salas, 2013).

In general, the N gene has been used for the detection of CDV by RT-PCR as one of the main targets for amplification, as it is a region conserved within the viral genome (Frisk *et al.*, 1999; López, 2012), which is corroborated in recent studies considering the complete nucleotide sequencing of a CDV viral strain in China (Jiang *et al*., 2013) or in studies on molecular detection of CDV and other viruses (Silva *et al*., 2014). In the Faculty of Veterinary and Animal Sciences of the University of Chile, several works have been carried out that involve most of the genes that make up the genome of the virus. Thus, Muñoz (2013), implemented a PCR for the N gene, and Jara (2011) and Salas (2013) targeted the H gene, achieving the first with the technique implemented, a 90% positive detection of the virus, versus 19% and 7% respectively, so the techniques used by Jara (2011) and Salas (2013) would be less sensitive. Mateo (2015), using the gene P and PINcheira (2015), using the L gene, achieved - for the established conditions of their work - a 100% positive detection of the virus, being effective the RT-PCR implemented as diagnostic method, what in the case of PINcheira (2015) contrasts with what was stated in relation to the fact that, in the

identification of the virus, more conserved genes are preferred, such as the N, P and M genes. In the case of the F gene, as indicated in his work Vera (2014), the variable region of the gene, Fsp, is a good indicator to perform CDV phylogeny.

In this study, the design of the starters was successful, since the RT-PCR implemented managed to detect the M gene in 14/20 of the RNA samples analyzed (70%) and in 100% of the vaccine controls. No nonspecific fluorescent DNA bands were observed, nor were bands found in the negative controls. An explanation of the results could consider that RNA samples stored at -20 ° C could be degraded. However, these samples were reused for the detection of the N gene and were positive (data not shown). On the other hand, the determination of nucleotide identity indicates that the fragment obtained is compatible with the detection of the M gene at a value of NIP = 98%, which corroborates the specificity of the primers used. Although these results are not optimal, they allow completing the study of CDV genes as a detection target using molecular detection and unfortunately, apparently, there are no previous studies using the M gene in the literature to be able to compare our results. Taking into account the results shown, it seems that the detection by RT-PCR must necessarily involve the N, L or P gene (Muñoz, 2013; Pincheira, 2015; Mateo, 2015). However, the above should still be demonstrated, considering some sensitivity test such as making dilutions in base 10 (for example) and determining the limit of detection, or standardizing the method used, since one of them (Mateo, 2015) considers a Nested PCR.

CONCLUSIONS

The primers designed for the RT-PCR test using the M gene as target, were effective in the detection of CDV, since they originated unique and sharp bands that allow to detect the virus in 14/20 samples positive to CDV (70%) With the obtained results, the determination of the optimal protocol of viral detection by means of RT-PCR would involve the N, P and L genes, since they surpass 90% of detection according to the samples analyzed. With this, the necessary measures could be taken for the prevention, treatment and control of the disease in a timely manner.

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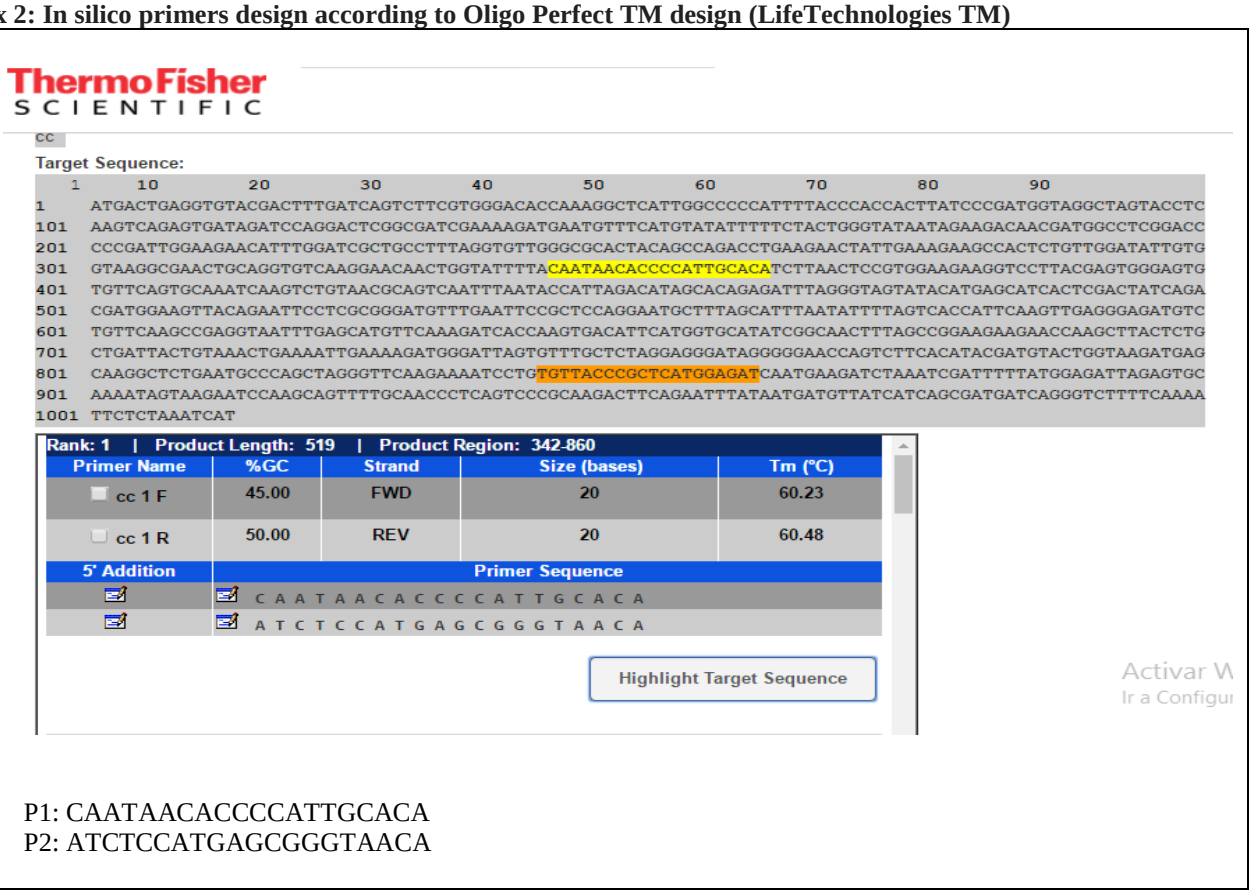
Annex 1: Consensus sequence from alignment through the OMEGA Clustal program, used for the primers design

ACTGAGGTGTACGACTTTGATCAGTCTTCGTGGGACACCAAAGGCTCATTGGCCCCCATTTTAC
CCACCACTTATCCCGATGGTAGGCTAGTACCTCAAGTCAGAGTGATAGATCCAGGACTCGGCG
ATCGAAAAGATGAATGTTTCATGTATATTTTTCTACTGGGTATAATAGAAGACAACGATGGCCT
CGGACCCCCGATTGGAAGAACATTTGGATCGCTGCCTTTAGGTGTTGGGCGCACTACAGCCAG
ACCTGAAGAACTATTGAAAGAAGCCACTCTGTTGGATATTGTGGTAAGGCGAACTGCAGGTGT
CAAGGAACAACTGGTATTTTACAATAACACCCCATTGCACATCTTAACTCCGTGGAAGAAGGTC
CTTACGAGTGGGAGTGTGTTCAGTGCAAATCAAGTCTGTAACGCAGTCAATTTAATACCATTAG
ACATAGCACAGAGATTTAGGGTAGTATACATGAGCATCACTCGACTATCAGACGATGGAAGTT
ACAGAATTCCTCGCGGGATGTTTGAATTCCGCTCCAGGAATGCTTTAGCATTTAATATTTTAGT
CACCATTCAAGTTGAGGGAGATGTCTGTTCAAGCCGAGGTAATTTGAGCATGTTCAAAGATCA
CCAAGTGACATTCATGGTGCATATCGGCAACTTTAGCCGGAAGAAGAACCAAGCTTACTCTGC
TGATTACTGTAAACTGAAAATTGAAAAGATGGGATTAGTGTTTGCTCTAGGAGGGATAGGGGG
AACCAGTCTTCACATACGATGTACTGGTAAGATGAGCAAGGCTCTGAATGCCCAGCTAGGGTT
CAAGAAAATCCTGTGTTACCCGCTCATGGAGATCAATGAAGATCTAAATCGATTTTTATGGAGA
TTAGAGTGCAAAATAGTAAGAATCCAAGCAGTTTTGCAACCCTCAGTCCCGCAAGACTTCAGA
ATTTATAATGATGTTATCATCAGCGATGATCAGGGTCTTTTCAAAATTCTCTAAATCAT

Annex 2: In silico primers design according to Oligo Perfect TM design (LifeTechnologies TM)



P1: CAATAACACCCCATTGCACA
P2: ATCTCCATGAGCGGGTAACA

Annex 3. nucleotide identity percentage of MG1C and MG2C, according to BLAST.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Descri	Max score	Total score	Query cover	E value	Ident	Accession
Canine distemper virus genomic RNA, complete sequence, strain: 50Cbl/H	893	893	100%	0.0	99%	AB490678.1
Canine distemper virus M, F, H, L genes for matrix protein, fusion protein, hemagglutinin,	893	893	100%	0.0	99%	AB476403.1
Canine distemper virus genomic RNA, complete sequence, strain: 50Con	893	893	100%	0.0	99%	AB476402.1
Canine distemper virus genomic RNA, complete sequence, strain: 007Lm/B	887	887	100%	0.0	99%	AB490680.1
Canine distemper virus genomic RNA, complete sequence, strain: 007Lm/H	887	887	100%	0.0	99%	AB490679.1
Canine distemper virus genomic RNA, complete sequence, strain: 50Con/H	887	887	100%	0.0	99%	AB490676.1
Canine distemper virus genomic RNA, complete sequence, strain: 011C/H	887	887	100%	0.0	99%	AB490674.1
Canine distemper virus genomic RNA, complete sequence, strain: 009L/H	887	887	100%	0.0	99%	AB490672.1
Canine distemper virus genomic RNA, complete sequence, strain: 55L/H	887	887	100%	0.0	99%	AB490670.1
Canine distemper virus viral cRNA, nearly complete genome, strain: 007Lm-H358p8	887	887	100%	0.0	99%	AB823707.1

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Canine distemper virus genomic RNA, complete sequence, strain: 50Cbl/H	881	881	100%	0.0	99%	AB490678.1
Canine distemper virus M, F, H, L genes for matrix protein, fusion protein, hemagglutinin, large p	881	881	100%	0.0	99%	AB476403.1
Canine distemper virus genomic RNA, complete sequence, strain: 50Con	881	881	100%	0.0	99%	AB476402.1
Canine distemper virus genomic RNA, complete sequence, strain: 007Lm/B	876	876	100%	0.0	99%	AB490680.1
Canine distemper virus genomic RNA, complete sequence, strain: 007Lm/H	876	876	100%	0.0	99%	AB490679.1
Canine distemper virus genomic RNA, complete sequence, strain: 50Con/H	876	876	100%	0.0	99%	AB490676.1
Canine distemper virus genomic RNA, complete sequence, strain: 011C/H	876	876	100%	0.0	99%	AB490674.1
Canine distemper virus genomic RNA, complete sequence, strain: 009L/H	876	876	100%	0.0	99%	AB490672.1
Canine distemper virus genomic RNA, complete sequence, strain: 55L/H	876	876	100%	0.0	99%	AB490670.1
Canine distemper virus viral cRNA, nearly complete genome, strain: 007Lm-H358p8	876	876	100%	0.0	99%	AB823707.1