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Original Research Article

Molecular Detection of Distemper Canine Virus Through the Use of the Large Polymerase Gene

Pincheira, D., Céspedes, P, Pizarro, J., Navarro C*
Medicine Preventive Departament, Faculty of Veterinary Medicine and Animal Sciences, University of Chile, Chile

*Corresponding Author
Navarro C
Email: canavarr@uchile.cl

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Abstract: The Canine Distemper Virus (CDV) is a widespread responsible for the Canine Distemper Disease that affects a broad diversity of carnivore mammals (and some cases in primates have been reported) globally. Domestic dogs are extensively and aggressively (with a ~50% mortality rate) infected by the CDV. Therefore, the clinical impact of the CDV is remarkable. The CDV is encoded by six genes, N, P/V/C, M, F, H and L. Almost all of which have been previously studied for lineage identification and disease diagnosis. In this study we used Reverse Polymerase Chain Reaction tests (RT-PCR) to analyseobtain 22 fragments of Ribonucleic acid (RNA) from peripheral blood of 22 subject dogs clinically diagnosed with CDV and positive to CDV by RT-PCR (N gene). The aim of this study was to implement a protocol of detection of the Large Protein Gene (L) to be employed as an alternative diagnostic. To achieve this, a pair of primers that generated a fragment of deoxyribonucleic acid (DNA) of ~450 base pairs (bp) was designed, which, using a bioinformatic analysis, confirmed its identity (NIP > 94%). Therefore, the implementation of this protocol opens the discussion about the best alternative method, within the context that the detection of the P gene through Nested RT-PCR is currently the best available protocol. A sensitivity analysis may potentially elucidate this question. Consequently, the main conclusion is that the molecular technique implemented in this study offers an effective diagnostic method to optimize the detection of VDC in domestic dogs.

Keywords: L gene, canine distemper virus, primer design, polymerase chain reaction

INTRODUCTION

Canine Distemper Virus affects a wide variety of species, most families of terrestrial carnivores such as *Canidae, Mustelidae* and *Procyonidae* (Appel and Summers, 1999), *Ailuridae, Hyaenidae, Ursidae, Viverridae* (Appel and Summers, 1995), large felines (Appel *et al.,,* 1994), marine carnivores such as the caspian seal (Phoca caspica) reported by Kennedy et al in the year 2000 and its presence in non-human primates has even been documented (Sakai *et al*,.2013).

The VDC genome has 6 genes whose function is to code the six structural proteins of the virion. These genes are N, P, M, F, H, L, each of which is responsible for coding a single protein, with the exception of P C that encodes phosphoprotein structural P and non-structural V and C (Lamb and Parks, 2007).

The gene H is the one with the greatest genetic variability and therefore it is used in different characterization studies of strains and lineages of the virus (Martella *et al.*, 2006), there are studies of its detection by polymerase chain reaction associated with retrotranscription (RT-PCR) as Jara does in 2011. It has also been used successfully for the determination of new viral genotypes in dogs in Japan (Mochizuki et al, 1999) as well as for the identification of at least two genotypes of the virus present in Chile. (Salas, 2013). For the diagnosis of the disease, RT-PCR is usually used on the N gene (Alcalde *et al.*, 2013, Muñoz, 2013).

The main objective of this study is to achieve the detection of the gene for the large protein (L) of the canine distemper virus using RT-PCR, through the design of the primers needed for the reaction, the amplification of a DNA fragment 450 base pairs (bp) and finally the establishment of the nucleotide identity of the obtained fragment.

MATERIAL AND METHODS

Samples: Two strains of VDC from commercial vaccines (Lederle and Onderstepoort) were used as positive controls for the RT-PCR reaction. For the analysis, 22 samples of viral RNA obtained from peripheral blood of dogs of different breeds, ages and vaccination status against VDC were used, with clinical

signs of DC and positive to RT-PCR on the N gene. As a negative control, two samples of RNA extracted from dogs without DC signology and negative to RT-PCR were used on the N gene. As control of reagents, nuclease-free water was used.

Primer design: The primers for the reaction were obtained by using the free access online program OligoPerfectTM Designer from Life Technologies™. For this, the nucleotide sequence of the region comprising the nucleotide 9030 to 15584 corresponding to the gene of the large protein L available in GenBank® was entered the program to obtain a DNA fragment of approximately 450 bp (annex 1). Once the primers were designed, they were sent to Bioscan® to be synthesized. **RT-PCR**: For the implementation of this technique an Apollo (CLP, USA) thermocycler with 96 wells of 0.2 mL was used and a protocol that contemplated denaturation at 94 ° C for one minute, alignment at 55 ° C for one minute, elongation at 72 ° C for one minute and final elongation at 72 ° C for seven minutes. **Reaction mixture**: 15 µL of the commercial kit "SuperScript one step RT-PCR with platinum taq" (Taq DNA polymerase, MgCl2 and the deoxyrubonucleotides trifostatos), 5 µL of the template DNA and 5 µL of each specific primer (designated P1) were used. and P2 respectively), reaching a final volume of 30 µL. **DNA amplification**: The PCR technique contemplates a DNA denaturing step, followed by a stage of alignment of the primers and a final stage of elongation to finish a cycle. Through a thermal gradient thermocycler (Px2 Thermal Cycler), the optimum alignment temperatures for the pair of primers were determined: 55 ° C. After 35 cycles, the amplified product was visualized.

Detection of amplified products: The detection of the amplified product was carried out by electrophoresis in 2% agarose gel (Winkler®) in Tris acetate EDTA (TAE) buffer (Fermentas®) and subsequent staining with ethidium bromide (0.5 µg/mL) (Fermelo®). Thus, 5 µL of the PCR product was taken and mixed with 1 µL of a commercial loading product, 6X Mass Ruler Loading Dye Solution (Fermentas®), which has glycerol to provide sample density and bromophenol blue to verify the progress of migration of the DNA bands. The electrophoresis was carried out at 90V for 90 minutes. As a molecular size marker, Hyperladder I (Bioline®) was used, which contains fragments of DNA between 50 and 1000 bp. With this product the size of the amplified fragments was compared. At the end of the electrophoresis, the bands were visualized in a transilluminator of ultraviolet (UV) light (Transiluminator UVP®) and photographed with a digital camera and a suitable filter.

Determination of the nucleotide identity of the amplified fragment.

a) **Sequencing**. Three positive samples were sequenced 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 Ltda.

b) **Nucleotide identity percentage (NIP).** The sequences delivered by Genytec Ltda. Were aligned to obtain a consensus sequence for each sample, through the Clustal Ω online program. The consensus sequences were entered into the BLAST sequence alignment computer program, to know their nucleotide identity with respect to the first 15 results delivered by Clustal Ω.

Analysis of results: Any one that originated a fragment of DNA of approximately 450 bp and NIP higher than 90% was considered as a positive sample. Biosecurity measures: Biosecurity measures were used long and closed sleeve apron, latex gloves for the handling of potentially harmful or toxic products. When using the ultraviolet light transilluminator an acrylic plate was used as a protective filter and the gels used were wrapped in latex gloves, deposited in a specially destined container and subsequently incinerated, while the material contaminated with virus was sterilized by autoclave.

RESULTS

Design of the primers required for the reaction

The primers designed by the OligoPerfect™ Designer program of Life TechnologiesTM were designated PGL1 F: 5'-ACTCACAACTGTCGCAAACG-3' and PGL1 R: 5'-TTGGGTTAAGGTTT GGAACG-3 '(Annex 1).

Amplification of the 450 bp DNA fragment.

The visualization of the 2% agarose gel in the UV light transilluminator allowed to determine the presence of unique and clear fluorescent bands around 450 bp. in the positive controls and in the total of positive samples by RT-PCR to the VDC N gene. No fluorescent bands were observed in the negative controls or in the control of reagents. In figure 1 it is possible to observe the DNA fragments synthesized in the RT-PCR reaction of the different samples and controls.

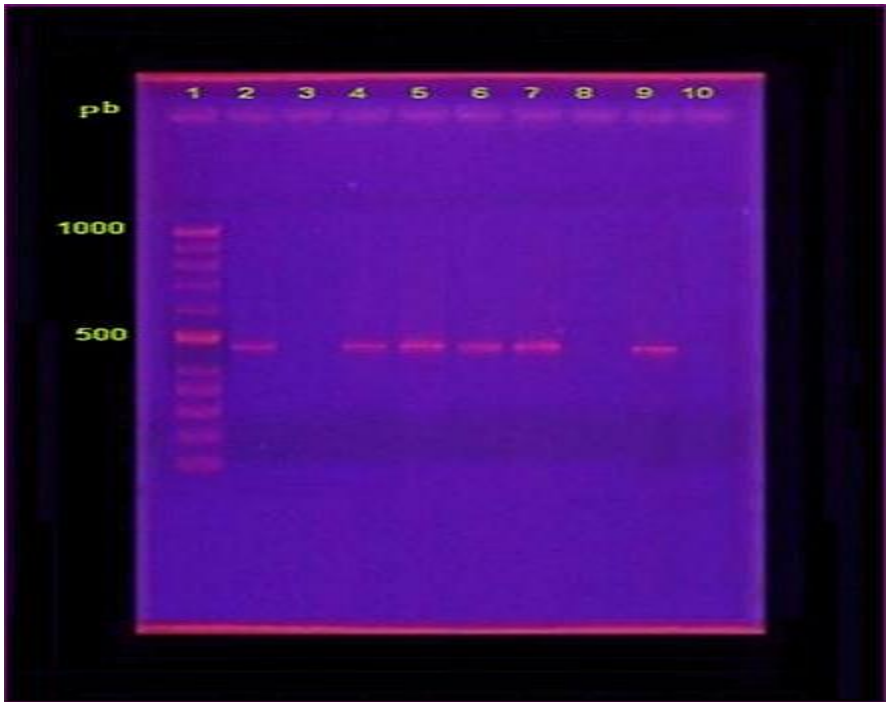

Figure 1: RT-PCR of the VDC L gene of positive and negative samples of the virus. Lane 1: MTM (molecular size marker 100 to 1000 bp). Lane 2: VDC (Lederle strain). Lane 3: negative sample. Lane 4: sample 1. Lane 5: sample A. Lane 6: sample H. Lane 7: sample F. Lane 8: negative sample. Lane 9: VDC (strain Onderstepoort). Lane 10: reagent control

Determination of the Nucleotide Identity Percentage (NIP)

a) Sequencing: Of the 22 samples from dogs positive for CDV that generated a fluorescent band with a molecular size of around 450 bp, those coming from individuals A, F and H were sent in triplicate to sequencing. The sequences received from Genytec Limited was entered to the Clustal Ω online program, a consensus sequence was obtained for each trio of sequences. Additionally, by aligning the consensus sequences with each other, the intersection identities were determined.

b) Percentage of nucleotide identity (NIP): The individual entry of the consensus sequences entered the BLAST online program allowed to determine the nucleotide identity of each one, with respect to the first 15 results delivered by Clustal Ω. Additionally, as an example, each sequence was aligned with one of the strains delivered by Blast. randomly chosen, indicating only nucleotide identity with respect to VDC strain data.

DISCUSSION

The Canine Distemper is a contagious and, in many cases, fatal disease that affects several families of mammals, mainly carnivores (Appel and Summers, 1999), being produced by the Canine Distemper Virus. For identification there are different diagnostic techniques, including the polymerase chain reaction associated with retrotranscription (RT-PCR). To this end, most of its genes have been used as targets, however, given the impact on both domestic and wild animals that the VDC infection produces, it is necessary to establish an increasingly better diagnostic technique, which implies the study of all its genes as a target to determine which of them results in a better RT-PCR. Recent studies maintain the N gene as the gene of choice (Jiang *et al*.,2013; Silva *et a.,l* 2014) for the complete nucleotide sequencing of a viral strain of VDC in China or in molecular detection of VDC and other viruses, or the use of the H gene, both for the determination of 8 VDC lineages (Sarute *et al.*, 2013) as well as for the identification of the "Arctic" lineage in an outbreak of DC in Italy (Di Sabatino *et al.*, 2014), among others. To date, there are no studies that use the gene for the large L protein as a target, and this research is attractive. The present study determined that for the established conditions, RT-PCR is effective on the L gene as a diagnostic method for the detection of VDC.

Thus, from 22 RNA samples analyzed, all 22 tested positive to VDC, which corresponds to 100%. In contrast to other RT-PCR studies for VDC conducted in the same Faculty of Veterinary and Animal Sciences, using the H gene as a target, they showed positive values of 7% (Salas, 2013) and 83% (Jara, 2011), and about the N gene 91% (Muñoz, 2013). These results may suggest a greater sensitivity of RT-PCR on the L gene compared to other genes.

As for the positive and negative controls, when visualizing the 2% agarose gel in the UV light transilluminator, both positive controls showed clear fluorescent bands of 450 bp while both negative controls, did not yield bands for any molecular size. These results could venture in the first instance a high specificity of the method established.

Under light this results, a high sensitivity and specificity of the RT-PCR could be assumed on the L gene, however to confirm such a claim, further studies are required oriented to diagnostic tests that determine validity parameters such as sensitivity and specificity, parameters that were not evaluated in this study, nor were efficacy parameters evaluated. It should be noted that of the 22 samples analyzed and that finally were positive, 12 of them came from dogs vaccinated against VDC, which corroborates studies that indicate that vaccination does not generate a completely effective immunity (Appel and Summers, 1999; Gemma *et al.*, 1996), which suggests the existence of important antigenic differences between VDC isolates from different geographical areas with vaccine strains, which could be responsible for the resurgence of the disease (Harder and Osterhaus, 1997). Although the present study suggests that RT-PCR of the L gene is a good diagnostic test for CDV, it does not determine parameters of validity (specificity and sensitivity) or efficacy (positive predictive value and negative predictive value), which should be evaluated in later studies. Having confirmed the effectiveness of RT-PCR on the L gene, contributes greatly to broadening the knowledge about molecular diagnosis on VDC. The results of this work, plus the previous results obtained using other VDC genes, lead to the need to perform future comparative studies between RT-PCR reactions, using as target the 6 VDC genes. Establishing for each of them parameters of validity and efficacy that allow in this way, determine which of them constitutes a better diagnostic test for the VDC.

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Anexo 1: Diseño de partidores según OligoPerfect™ Designer de *Life* Technologies™

Rank: 1 | Product Length: 457 | Product Region: 1404-1860

Primer Name	%GC	Strand	Size (bases)	Tm (°C)
PGL 1 F	45.00	FWD	20	59.95
PGL 1 R	55.00	REV	20	59.98

5' Addition	Primer Sequence
	T T G G C A G C C C T A A G A A A A G A
	C T C G A T A A G G T G C A G G C T T C