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Research Article

Canine Distemper Virus: Multiple Detection of the H and N Genes by the Polymerase Chain Reaction Associated with Reverse Transcriptase

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Abstract

Canine Distemper Virus (CDV) is a virus that has several potential hosts, all carnivores, which can spread to wild animals, increasing their predisposition in winter. It has a high prevalence and is highly contagious, being one of the main causes of death in domestic and wild canids. Although it is a monotypic virus, that is, with little genetic variability, the H gene has high genetic variation, which would produce different strains of the virus, some more virulent than others and with different tropism. It is because of this variability that patients get sick anyway, since the eightfold vaccine protects against a specific strain of the virus, America 1. So any carnivore susceptible to the virus that faces a strain against which it was not vaccinated has as many chances of getting sick like an animal that was simply never vaccinated against this viral disease. For this reason, it is essential to know which are the lineages of the virus present in the country and for that a diagnostic technique is necessary that not only tells whether the virus is or is not Canine Distemper, but also reveals whether it is one of the two lineages that already exist. knows that they are present or there are others. For this, it is necessary to carry out a multiple RT-PCR protocol, detecting the N gene, which is the least variable, and in parallel the H gene (America 1 and European lineage) to perform a phylogenetic analysis based on official and known sequences of the virus.

Thus, the general objective was the use a genomic database in conjunction with the in silico design of primers for the generation of an RT-PCR protocol capable of detecting canine distemper virus and the genotype involved, obtain sequences from GenBank of the CDV genome for gene N, America 1 and European, alignment of sequences with the Clustal W program and generation of consensus sequences and design of rimers using the OligoPerfect program.

Background

Canine Distemper Virus (CDV) is a lipid-enveloped pleomorphic virus belonging to the genus *Morbillivirus* of the *Paramyxoviridae* family, *Mononegavirales* order, and is antigenically related to the Rinderpest and Measles viruses. As the name of its order indicates, it is a negative sense single-stranded RNA virus with a helical nucleocapsid. Its genome, not segmented, consists of about 15.7 kilobases (kb) [1]. The genome of this virus encodes six structural proteins: the nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the polymerase (L) and the envelope glycoproteins: the fusion protein (F) and hemagglutinin (H). Proteins N, P and L together with viral RNA form the ribonucleoprotein complex (RNP), which directs the sequential synthesis of mRNA from viral genes and the replication of antigenomes. Proteins F and H are integral proteins of the lipid envelope membrane, associated with protein M, which carries out the interaction between the RNP complex and the membrane. In addition to this, the integral proteins of the membrane are responsible for the recognition of the virion for entry to the host cells, being the main targets of the immune system [2,3]. *In vitro* studies have also shown that the hemagglutinin protein is the main determinant of cell tropism [4]. It is an enveloped virus, so it is very sensitive to the environment, however, its constant elimination through retions and body fluids from the seventh day after infection and its high infectivity, allow its rapid dissemination in the ecosystem. In addition to this, there are infected animals that shed the virus before showing associated signs [2].

Hosts range

The CDV has numerous hosts, all of them belonging to the order of carnivores such as *Canidae* (dogs, foxes, wolf, dingo, coyote), *Procionidae* (raccoons, coati), *Mustelidae* (ferrets and minks), *Felidae* (tigers, lions, leopards) and even in marine mammalian animals [2, 5-7]. On the other hand, a possible relationship between Paget's disease in humans and CDV infection has been demonstrated, due to the detection of viral RNA in affected tissues [8,9]. In Chile, in 1994 the first isolation of the virus in cell cultures was reported inoculated with retions from a canine with clinical signs of CD. Clinical diagnosis it was confirmed by electron microscopy and histopathological studies [10]. In 2003, an outbreak of DC occurred in the endemic fox populations of the Fray Jorge National Park (Coquimbo Region), which it was speculated could be related to the existence of native mustelids such as chingue and quique [11]. Furthermore, four years later, in 2007, there was an outbreak of CD on Robinson Crusoe Island (Valparaíso Region), where several domestic dogs were affected, but not the endemic sea lions of the island [12].

Pathogeny

The course of the disease is variable, depending largely on the efficiency of the host's immune response. The main affected are puppies between three and six months of age, since their immune system is poorly developed and they have already lost maternal antibodies [2,7]. The virus enters the respiratory tract and during the first 24 hours it affects the regional lymph nodes and after 7 days all the lymphoid tissue, producing an immunosuppression due to the decrease in the proliferation of T and

B-lymphocytes [3]. In the following weeks, the animal may recover or have symptoms associated with a secondary viremia, since the infected mononuclear cells transport the virus to the epithelial surface of the digestive, respiratory, urogenital, skin and/or central nervous system, with the respective clinical signs of each affected organ [5]. There is currently no effective treatment against CDV, in addition to antibiotic treatment to avoid secondary infections, which are highly frequent in immunosuppressed animals [7]. Most of the vaccines used correspond to polyvalent attenuated virus vaccines, which confer limited protection to individuals and also have the risk of causing disease in them, because they maintain their lymphotropism and ability to induce immunosuppression [7,13]. Recombinant vaccines are a safer option since they dispense with the pathogen and use some of its antigens to stimulate an immune response from the host. These vaccines show great efficacy, with a higher affinity and duration of antibody production than attenuated virus vaccines [14].

Diagnostic methods

There is a wide variety of laboratory diagnoses that can be carried out to differentiate them from other diseases with similar symptoms. Among them is:

i. **ELISA for the detection of specific IgM against CDV:**

This test is quite useful, since immunoglobulin M in dogs infected by CDV persists for 5 weeks to 3 months depending on the strain and the immune response of the host. Sampling should be at least 3 weeks after vaccination against the virus, since IgM persists around that time in the animal, which may result in a false positive [15].

ii. **Molecular techniques such as PCR:**

The Polymerase Chain Reaction associated with reverse transcription of the viral genome (RT-PCR) is used for the epidemiology of the virus and the circulation dynamics of the different existing strains. Using this technique and based on H gene sequencing, it has been possible to determine the existence of at least 14 different strains of the virus: Europe-1/South America-1, South America-2, South America-3, Wild Europe, Asia-4, America-2, Rockborn-Like, Africa-2, Asia-1, Arctic, Africa-1, Asia-2, Asia-3 and America-1 [13]. Of these lineages, it has been proven that in Chile, there are at least two of them, the America 1 and Europe-1/South America-1 lineage [16].

To update this information, a protocol was developed to make use of this diagnostic tool to determine the lineage of the strains that are currently present in our country, this being very important information for the development of a possible vaccine to protect pets. against CDV completely to prevent vaccinated dogs from getting sick anyway.

Materials and Methods

In the first instance, the accession numbers to the GenBank of previous work [17], were used to obtain the nucleotide sequences of the H gene in the America-1 and European-1 lineages, obtaining 12 sequences of the first and 34 of the second. Some of these sequences had the complete genome, for which it was possible to also obtain the sequence of the N gene in 5 of them, 4 from the America-1 lineage and 1 from the European-1 lineage. Despite being N gene a highly conserved gene, 10 more sequences were searched in GenBank, taking these randomly. Some of these were quite short, 2 of them did not exceed 300 base pairs (bp) and another 4 had a base pair number of less than 750 bp. All sequences and their respectives GenBank accession numbers will be included in the annexs. Once all the sequences of N and H gene (America-1 and European-1) were gathered, respectively, they were introduced to the open access program, Clustal Omega. This program automatically aligns the sequences and places an asterisk under the bases that are the same, being necessary for this that absolutely all the aligned bases are equal, since, if only one varies, the program does not place the asterisk. Initially, through this program, 3 different alignments were obtained, one for America-1, another for the European-1 lineage and finally a last for the N gene. These alignments will be in the annex. From each of these 3 resulting alignments a nucleotide sequence was chosen to be used in the open access program, OligoPerfect from ThermoFisher. The function of this program is, under certain parameters, to propose possible starters or primers for a PCR of a given sequence. The parameters that were varied were the included area, the excluded area and the length of the final fragment, these values being determined based on the previous alignments carried out in the Clustal W program (Tables 1-3). For each sequence, the parameters were the following:

Table 1: Parameters used for the design of the America-1 lineage primers.

Included sequence	88-1812
Excluded sequence	107-108, 129-184, 203-205, 296-314, 377-396, 424-426, 452-484, 506-567, 610-613, 643-723, 755-768, 793-794, 818-864, 893-991, 1028-1053, 1123-1158, 1193-1201, 1272-1274, 1311-1314, 1361-1472, 1517-1528, 1574-1602, 1623-1648, 1673-1674, 1710-1715, 1741-1750
Fragment size	450-550
Accession number	AY548109

Table 2: Parameters used for the design of European-1 lineage primers.

Included sequence	21-1756
Excluded sequence	90-92, 119-161, 202-206, 233-280, 300-301, 336-356, 397-413, 467-520, 546-837, 877-896, 916-963, 985-1058, 1085-1090, 1113-1121, 1159-1404, 1450-1482, 1517-1709
Fragment size	950-1050
Accession number	Z77673

Table 3: Parameters used for the design of N gene primers.

Included sequence	2-225
Excluded sequence	32-225
Fragment size	undetermined
Accession number	KU983567

For the first two, those fragment sizes have been chosen respectively, since the temperatures are less variable between the two starters, in addition all% GC are above 50%. This is important, since this is how the fragments will be differentiated in the electrophoresis, after the RT-PCT is performed (Tables 4 & 5). The resulting starters were as follows:

Table 4: Primers generated from sequence AY548109 (America-1).

Size (bp)	Primers Sequence	%GC	Tm (°C)	ΔTm (°C)
466	AGGTGGCTGAATGACATGCC GAGGTAATGTCAACCGCCCA	55,00 55,00	60,68 60,04	0,64
474	ACACTGGCTTCCTTGTGTGT TCCGGAGTTCAAAAGTGGGC	50,00 55,00	59,74 60,54	0,8
473	ACACTGGCTTCCTTGTGTGT CCGGAGTTCAAAAGTGGGCT	50,00 55,00	59,74 60,54	0,8
483	ACACTGGCTTCCTTGTGTGT GGTAAGCCATCCGGAGTTCA	50,00 55,00	59,74 59,46	0,28
484	ACACTGGCTTCCTTGTGTGT TGGTAAGCCATCCGGAGTTC	50,00 55,00	59,74 59,46	0,28

Table 5: Primers generated from sequence Z77673 (Europe-1).

Size (bp)	Primer Sequence	%GC	Tm (°C)	ΔTm (°C)
955	TACCAAGACAAGGTGGGTGC TCCCCAGCGTCACTACTAGA	55,00 55,00	59,89 59,38	0,51
951	AAGACAAGGTGGGTGCCTTC TCCCCAGCGTCACTACTAGA	55,00 55,00	60,18 59,38	0,8
955	TACCAAGACAAGGTGGGTGC TCCCCAGCGTCACTACTAGAA	55,00 52,38	59,89 59,99	0,1
951	AAGACAAGGTGGGTGCCTTC TCCCCAGCGTCACTACTAGAA	55,00 52,38	60,18 59,99	0,19
954	TACCAAGACAAGGTGGGTGC CCCCAGCGTCACTACTAGAA	55,00 55,00	59,89 58,53	1,36

In the case of N gene, the program did not show starters, since, when reviewing the included and excluded sequence values, it highlighted that only one sequence of the entire sequence has an amount greater than or equal to 20bp, so the design it was impossible. To solve this, the sequences were classified into 2 groups, one of them called "short sequences" that included 6 sequences that did not exceed 750 base wall and another 9 "long sequences", since these had about 1500 bp. The sequences and their classification are in the annex, together with the accession numbers. For these two sequence classifications, the corresponding alignments were carried out in the Clustal W program. These two alignments, which are also recorded in the annex, show a large number of asterisks, which shows that the N gene is indeed highly conserved. . Once the alignments were ready, they were evaluated to choose the parameters on which the design of gene N primers would be based. For this gene, a size of the resulting fragment of maximum 350 bp has been arbitrarily chosen since it could also be differentiable. of the above fragments in electrophoresis (Tables 6-11). The parameters chosen for use in the OligoPerfect program and the resulting starters are as follows:

Table 6: Parameters used for the design of primers in short sequences of N gene.

Included sequence	111-251
Excluded sequence	143-144,165-175
Fragment size	80-110
Accession number	KU725679

Table 7: Primers generated from sequence KU725679 (N gene). Note: These primers were not considered suitable, as their Guanine and Cytokine percentage is less than 50% in most cases.

Size (pb)	Primer Sequences	%GC	Tm (°C)
89	CCCCAGGGAACAAGCCTAGA AGTTAAGATGAAACTAGCTAATCCAGC	60,00 37.04	60,91 59,29
90	TCCCCAGGGAACAAGCCTAG AGTTAAGATGAAACTAGCTAATCCAGC	60,00 37.04	60,91 59,29
88	CCCAGGGAACAAGCCTAGAA AGTTAAGATGAAACTAGCTAATCCAGC	55,00 37.04	59,01 59,29
90	TCCCCAGGGAACAAGCCTA AGTTAAGATGAAACTAGCTAATCCAGC	57.89 37	59,84 59,29
88	CCCAGGGAACAAGCCTAGAAT AGTTAAGATGAAACTAGCTAATCCAGC	52,38 37.04	59,43 59,29

Table 8: Parameters used for the design of primers for Gene N gene (short sequences) without a determined size of the final fragment.

Included sequence	111-251
Excluded sequence	143-144,165-175
Fragment size	undetermined
Accession number	KU725679

Table 9: Primers generated from sequence KU725679 (N gene; short sequences) without a determined final fragment size.

Size (bp)	Primer Sequence	%GC	Tm (°C)	ΔTm
131	CCCCAGGGAACAAGCCTAGA CAACCCAAGAGCCGGATACA	60,00 55,00	60,91 59,75	1,16
132	TCCCCAGGGAACAAGCCTAG CAACCCAAGAGCCGGATACA	60,00 55,00	60,91 59,75	1,16
130	CCCAGGGAACAAGCCTAGAA CAACCCAAGAGCCGGATACA	55,00 55,00	59,01 59,75	0,74
132	TCCCCAGGGAACAAGCCTA CAACCCAAGAGCCGGATACA	57.89 55,00	59,84 59,75	0,09
130	CCCAGGGAACAAGCCTAGAAT CAACCCAAGAGCCGGATACA	52,38 55,00	59,43 59,75	0,32

Table 10: Parameters used for the design of primers for N gene (long sequences) with a resulting fragment size of 250 to 350bp.

Included sequence	91-1205
Excluded sequence	117-213, 246-252, 280-281, 312-315, 336-367, 426-543, 576-621, 679-724, 768-769, 795-849, 870-1014, 1044-1179
Fragment Size	250-350
Accession number	AB932517

Table 11: Primers generated from sequence AB932517 (N gene; long sequences) with a final fragment size of 250 to 350bp.

Size (pb)	Primer Sequence	%GC	Tm (°C)	ΔTm
340	GAAGGGTCGAAAGCTCAAGG TAGGCTTGTTCCCTGGGGAT	55,00 55,00	58,56 60.25	1.69
345	ATCCTCTCCTTGTTCGTGGA GTCGGCTGCAGTATCAGGAG	50 60,00	58.06 60.25	2,19
347	ATCCTCTCCTTGTTCGTGGA GAGTCGGCTGCAGTATCAGG	50 60,00	58.06 60.25	2,19
341	GAAGGGTCGAAAGCTCAAGG CTAGGCTTGTTCCCTGGGGA	55,00 60,00	58,56 60,91	2,35
342	GAAGGGTCGAAAGCTCAAGG TCTAGGCTTGTTCCCTGGGG	55,00 60,00	58,56 60,91	2,05

Due to the difference in temperatures Tm, the analysis of the primers that would be used was carried out, focusing for Gene N on primers designed with the parameters expressed in Table 8. Finally, the chosen primers are marked in bold in their respective tables. The criteria for choosing them were the % GC, seeking to be as high as possible and the Melting Temperature (Tm) as less variable as possible within and between the different gene primers (Table 12).

Table 12: Melting temperature and their respective averages to calculate the total average of the Melting temperature.

Gen/Linage	Tm	ΔTm	Average Tm
América-1	59,74; 59,46	0,28	59,6
Europa-1	59,89; 59,99	0,1	59,9
Gen N	59,84; 59,75	0,09	59,8
			59,8

Thus, with the average of all Melting temperature (Tm) is possible to calculate the alignment temperature (Ta) under the following theoretical formula: Ta=(Tm-5), which can be corroborated with a temperature gradient thermal cycler.

Discussion

The Canine Distemper Virus is a frequent consultation in veterinary clinics, since in recent years dogs that are vaccinated become ill and it is no longer an incidental disease in puppies, but now affects any age range. That is why it is important to know a protocol to detect the presence of a third strain of the virus present in the country. This information would be useful to manufacture in the future a vaccine that does not protect only for the American strain. It is already known that there is at least one more strain, the European one, and this of course gives way to any other of the many strains present in the world, especially with the large number of trips abroad that are made in company. of our pets. On the other hand, the details of the protocol, for example, times and temperatures will be determined when testing the protocol, since only the alignment temperature has been obtained and not the others, since these are determined by the manufacturer. In this sense, it would be interesting to try a PCR with a temperature gradient to see if the chosen temperature (Ta) is the best or would there be one where a greater amount of product is produced and, therefore, the protocol has a better effectiveness.

Conclusion

It has been possible to create a protocol that allows updating the phylogenetic analysis of the CDV strains present in our country, this being of great importance for the epidemiological surveillance of the virus and for the possible creation of a vaccine that is more efficient against this virus by not cover only one of the strains. The scale is that the protocol can not only check if a sample is positive or negative for the virus, evaluating the presence of the conserved N gen, but it can also say in parallel the lineage to which it belongs on occasions, but in case of not being able to demonstrate it , this would dictate that it is a different lineage from the two known.

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