Open Access Research Journal of
Multidisciplinary Studies
Journals home page: https://oarjpublication/journals/oarjms/
ISSN: 2783-0268 (Online)

(RESEARCH ARTICLE)

Detection of the canine herpes virus glycoprotein B gene via Polymerase Chain Reaction

Leidy Carrasco Madrid, María Antonieta Jara and Carlos Navarro Venegas *

Department of Preventive Animal Medicine. FAVET. Universidad de Chile.

Open Access Research Journal of Multidisciplinary Studies, 2022, 03(01), 042–052

Publication history: Received on 01 January 2022; revised on 05 Februay 2022; accepted on 07 Februay 2022

Article DOI: https://doi.org/10.53022/oarjms.2022.3.1.0031

Abstract

The *Canine Herpesvirus* type 1 (CaHV-1) causes fatal hemorrhagic disease in puppies less thanfour weeks, while in four weeks older puppies it produces some minor clinical signs such as rhinitis, pharyngitis, or conjunctivitis. In adults, is capable to producing eye disease and reproductive disorders, and it is a participant pathogen called "kennel cough".

The gene coding for gB, a glycoprotein present in the viral envelope, is described in a great variety of herpes virus, because it is essential to the process of entry of virus into the cell and thusdefine the route of neuroinvasion.

This work was conducted in the laboratory of Virology and Microbiology, Department of Animal Preventive Medicine, Faculty of Veterinary and Animal Sciences, University of Chile and its purpose was to contribute to the molecular characterization of isolated viral RP5, and confirm thepresence of a herpes native virus, by detecting the glycoprotein B gene by Polymerase Chain Reaction.

The results allow demonstrating a high sensitivity of the technique implemented and a high specificity than other PCR protocol implemented in other title memory. Thus, a high nucleotide identity percentage (NIP>98) compared to the sequence published in GenBank allows the qualification of this protocol as a serious contender to become a diagnostic method for CaHV-1 in Chile and on the other hand it concludes that the isolated native RP5 corresponds to CaHV-1.

Keywords: CaHV-1; Molecular diagnosis; RP5 isolate; GB detection

1. Introduction

Canine Herpesvirus type 1 (CaHV-1) was originally isolated from a case of sudden-onset mortality in a canine litter and described for the first time in the United States of America as the cause of a fatal hemorrhagic disease in puppies under 4 weeks [1]

In adults, CaHV-1 causes infections of the external mucosa of the genital tract and has been associated with mild vesicular vaginitis. In addition, it is one of the participating agents of the respiratory syndrome called “kennel cough”, which corresponds to tracheobronchitis [2]. Currently, its presence has been described worldwide [3, 4, 5, 6].

1.1. Classification

CaHV-1 belongs to the *Herpesviridae* family, *Alphaherpesvirinae* subfamily, Genus *Varicellovirus* [7], this family together with two others make up the order *Herpesvirales*, which currently includes 3 families, 4 subfamilies, 17 genera and 90 species [8].

* Corresponding author: Carlos Navarro Venegas; Email: canavarr@uchile.cl
Department of Preventive Animal Medicine. FAVET. Universidad de Chile.

The *Herpesviridae* family is composed of many viruses that cause a variety of diseases, both in humans and animals [9]. The *Herpesviridae* family is made up of four subfamilies: *Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae* and a fourth yet unnamed [7]. This division was originally established by biological characteristics, but is generally consistent with later molecular determinations, including nucleotide sequencing and phylogenetic analysis [10,8].

1.2. Virus structure

The genome of the virions of the *Herpesviridae* family consists of a linear double-stranded DNA molecule, with a size that ranges between 125 and 235 thousand base pairs (bp), with a molecular weight of $63x10^6$ daltons [11]. Surrounding the genome is a capsid with icosahedral symmetry (162 capsomeres) and an approximate size of 125 nm. Next is a complex of viral proteins, with a fibrillar structure, called the tegument. Finally, there is a lipid envelope, derived from the host cell, on whose outer surface there are small projections evenly distributed, such as proteins and glycoproteins. The final structure of the viral particle has a size that varies between 120 and 300 nm [2, 9].

1.3. Viral replication

The phases of this process are summarized in 5 stages: adsorption, penetration, nucleic acid and protein synthesis, assembly and finally release (lysis) [9,10].

Adsorption is characterized by its high specificity; the virus proteins interact with cell receptors, which determines the cells that are likely to be infected. However, attachment and even penetration of a susceptible cell does not guarantee viral multiplication, since for replication to occur, new copies of the viral genome must arise and virus-specific proteins must be synthesized [9]. In viral replication, three classes of mRNA are produced: immediate early (α) that encodes five regulatory proteins; the delayed early (β) that encodes proteins related to DNA replication, including thymidine kinase, and the late (γ) that encodes structural proteins of the viral particle. Synthesized proteins fall into two categories: early proteins, which are synthesized immediately after infection and are necessary for viral nucleic acid replication; and late proteins, those that are synthesized later and include the virus envelope glycoproteins [9,10].

The synthesis of the viral DNA and the capsid occurs inside the nucleus of the host cell and from which it acquires the envelope. The virus is transported through the endoplasmic reticulum and the Golgi apparatus to the cytoplasmic membrane of the host cell where it is released or remains inside in a state of latency [12].

1.4. Characteristics of infection

Canine herpesvirus is widespread throughout the world, especially in canine communities, shelters, dog hotels, pet shops and particularly in kennels; in the latter, seroprevalence can exceed 90% and half of seropositive animals have reproductive disorders [13].

The herpes virus virion is fragile and its low resistance is due to its glycoprotein and lipid envelope, which is easily destroyed by heat, ultraviolet rays, lipid solvents and common disinfectants [10]. Its multiplication is optimal between 32°C and 33°C, observing rapid replication with lysis of infected cells. However, it is easily inactivated at temperatures above 40ºC [1, 13]. Transmission requires close contact between individuals through mucous membranes: through intercourse, licking and caresses of the mothers. In confined populations (breeding grounds), aerosol spread is the main mode of transmission [10].

1.5. Pathogenesis

In newborns, the primary multiplication takes place in the nasopharyngeal mucosa and tonsils, followed by an invasion of the regional lymph nodes. Viremia is associated with mononuclear cells, followed by lymphoid hyperplasia and necrosis of the spleen and deep lymph nodes such as mediastinal, iliac and mesenteric. Infection of the vascular endothelium due to necrosis causes the appearance of lesions such as hemorrhages and scattered necrosis in the main organs [12, 14]. Puppies older than four weeks and adults are considered immunocompetent animals, since their body temperature is higher than that required for optimal virus multiplication, mainly in the upper respiratory tract and genital tract.

1.6. Associated diseases

1.6.1. Newborns under four weeks

CaHV-1 causes a brief but severe disease, characterized by viremia and 80% mortality in animals less than one year old. The severity of the disease is related to (a) inability to adequately regulate body temperature, as puppies at birth are

relatively immature with respect to hypothalamic thermoregulatory centers, (b) inability to mount a febrile response to infection, and (c) immaturity of their immune response.

Once this capacity develops (2-3 weeks of age), puppies become resistant to systemic infections, because the virus does not replicate efficiently at temperatures above 36°C. At the same time, immune maturity occurs in puppies, translated into the acquisition of some mechanisms of innate immunity: inhibition of infection by type I interferons (IFN) and the destruction of infected cells by natural killer (NK) cells [2, 15]. The few surviving animals are left with irreversible cerebellar and joint sequelae [2, 13].

1.6.2. Adults

CaHV-1 causes different pathological signs: eye conditions such as transient conjunctivitis and rhinopharyngeal conditions that manifest clinically as "kennel cough" [12, 13]. It is frequently associated with this syndrome, but it is considered a secondary agent, which has been demonstrated in its low detection rate (1.46%) like *Canine Coronavirus* and *Canine Distemper Virus* (1.5%), compared to *Canine Parainfluenza Virus* (7.4%) and *Bordetella bronchiseptica* (10.3%). With this, it can be concluded that since this disease is caused by many infectious agents, CaHV-1 is considered a secondary pathogen [16].

Reproductive disorders and genital lesions are the most important signs at this stage. In males, the presence of nodules in the penile mucosa is described, which becomes reddish and rough, with the appearance of petechiae and purulent discharge [13, 17].

1.7. Diagnosis

1.7.1. Clinical diagnosis

Clinical signs and necropsy provide elements of suspicion that must be confirmed by complementary laboratory tests. In a kennel, herpesvirus infection is suspected when perinatal mortality suddenly appears, followed by abortions, infertility, and sometimes kennel cough; hence the importance of having a history of the hatchery [13].

1.7.2. Laboratory diagnosis

Hematological and biochemical abnormalities. They are not specific but marked thrombocytopenia and a marked increase in Alanine-Aminotransferase (ALT) activity are seen in affected newborns [12, 13].

1.8. Viral isolation

It consists of achieving the multiplication of the virus in susceptible animals, embryonated eggs or cell cultures, obtaining as a result the presence or absence of a visible effect on its respective host [18]. It is difficult to achieve and requires optimal culture conditions, it generally multiplies in cells of canine origin, such as kidney cells [2]. For viral isolation, samples from newborns are preferably from the kidneys, liver, lungs, spleen, lymph nodes, and adrenal glands. In adults, it can be achieved from nasal, vaginal, or preputial swabs [12].

1.9. Necropsy

It is the systematic study of a dead animal directed to the search for lesions. It is an important diagnostic tool and compatible with other diagnostic procedures [19]. Postmortem, characteristic lesions such as hemorrhagic petechiae and scattered foci of necrosis are evident frequently in the liver, spleen, lungs, and kidneys [2, 13, 14]. Thus, they can also appear in the brain, heart, adrenal glands and intestine [14]. Splenomegaly and generalized lymphadenomegaly are common [12]. Histologically, pathognomonic lesions of herpesvirus can be observed, such as intranuclear inclusion bodies [12, 13].

To obtain good samples and their subsequent visualization, an early necropsy and immediate fixation in 10% formalin should be performed; samples of placenta, liver, lung and mainly kidney should be taken [13].

1.10. Serological methods

Several tests have been developed to detect antibodies against CaHV-1, among which we can mention: serum neutralization (SN), indirect immunofluorescence (IFI), hemagglutination inhibition (HAI) and ELISA [20]. SN is based on the blocking of viral multiplication by means of specific antibodies, which makes it possible to measure the ability of a serum to inhibit or neutralize the infectivity of a virus on a susceptible host [18]. A rapid diagnosis can be made through immunofluorescence (IF) from sections of affected tissue [2]. IF makes it possible to visualize, through a

fluorescence microscope, the antigen/antibody reaction by means of the fluorescence emitted by a labeled antiglobulin (antibodies directed against gamma globulins). IHA is based on the binding of antibodies to virus receptors for erythrocytes, preventing the virus from adsorbing to erythrocytes; This allows the identification of viruses with hemagglutinating capacity and evidence of antigenic differences between types of the same virus. Lastly, the ELISA test makes it possible to detect the union of an antigen and an antibody by means of a colorimetric reaction associated with the presence of an enzyme bound to the antibody. Thus, the color change of the substrate (absorbance) can be determined with a spectrophotometer [18].

1.11. Molecular detection

It can be performed by *Polymerase Chain Reaction* (PCR), a very useful technique in molecular biology. This sensitive and specific technique detects part of the genome of the pathogen of interest and can be used to detect viral DNA from affected organs [17, 21].

The PCR technique is a method to amplify nucleic acids, like the natural process of DNA replication. The number of molecules is amplified after the repetition of three successive steps (denaturation, alignment and elongation), which together form a cycle, under controlled temperature conditions that are gradually modified by the thermocycler.

Compared with the PCR technique, the sensitivity and specificity for virus isolation is 49% and 100%, respectively, in the case of indirect immunofluorescence it is 29% and 96%, respectively [22]. It is the most reliable method to rule out serological false negatives [13].

1.12. Prevention

Considering that subsequent infection because of latency cannot be controlled, the important thing is to avoid or limit the clinical development of the disease. The latter would be achieved through the potential reduction of the infection, that is, the decrease in viral propagation. Thus, the main prevention measure corresponds to the establishment of adequate hygiene, since CaHV-1 is inactivated with most of the common disinfectants. Another important protective measure is to avoid potentially stressful risk factors, such as the introduction of unknown dogs in a kennel or the administration of glucocorticoids (methylprednisolone acetate) in immunosuppressive doses, since these trigger viral recrudescence [12]. The latter has not been demonstrated in other canids such as foxes (*Vulpes vulpes*), in which the administration of glucocorticoids does not reactivate CaHV-1. Perhaps this observation could be explained by the fact that CaHV-1-carrying foxes do not transmit the virus by contact, which in turn could explain the low prevalence of CaHV-1 in wild foxes [23].

1.13. Treatment

The use of antivirals, in general, has proven to be ineffective because viral replication depends on the metabolism of the host cell, or because viruses can be affected by antivirals only during their replication cycle, which normally goes unnoticed in the case of acute infections. Many of them are not approved because they are toxic, and the approved ones have been authorized for human use and limited application in dogs and cats [24].

1.14. Glycoprotein B

Antigenic relationships among herpesviruses are complex, and members of the family share some antigens; however, the various species have different envelope glycoproteins [10].

During infection, the immune response is directed towards the viral envelope glycoproteins, as antibodies elicit both a humoral and a cellular response [25]. Through studies carried out on bovine herpes virus (BoHV-1), it has been shown that immunization with glycoprotein B (gB) can induce a protective response [26].

In *Herpes Simplex Virus* type 1 (HSV-1) it was found that when generating a mutant, in which gB is exchanged with that of pseudorabies virus (PrV), it is evident that PrV gB was able to functionally replace gB of HSV-1 in HSV-1 virions, which did not occur in the reverse way [27].

The gene that codes for gB is essential both for the entry into the target cell in infections by free virions or for cell-to-cell spread, as well as for the determination of the neuroinvasion route. This route begins with the entry of the virus into the nerve endings of the periphery, it is transported to the cell body where it replicates and spreads transneuronally thanks to the synapse [27].

The gB gene encodes a protein of 879 amino acids [25]. Although the size of the gB gene is 2640 bp [28], in this work, the detection of two fragments of the gB gene in the RP5 isolate was implemented, one of 1286 bp and another of 450 bp, by means of the technique of Conventional PCR, as an approach to a useful diagnostic tool in the detection of canine herpes virus in Chile.

1.15. Situation in Chile

The presence of CaHV-1 in Chile was suggested years ago by the presentation of perinatal death, close to 80% in a kennel, whose puppies at necropsy showed the presence of petechiae in the kidney and liver, as well as many intranuclear inclusion bodies. eosinophils. The infection of cultures of the equine kidney cell line presented the characteristic cytopathic effect of herpes virus [29]. Later, in 2002, the presence of CaHV-1 was detected, obtaining a national isolate called RP5. When this isolate was inoculated into cell monolayers, both in primary lung and kidney cultures, and in the Madin-Darby Canine Kidney (MDCK) cell line, the typical cytopathic effect of cell lysis was produced at short times, starting from the third passage.

The direct immunofluorescence test in cell monolayers inoculated with the RP5 isolate, showed a high reaction between the commercial monoclonal antibody and the viral antigens present in the infected cells; thus, confirming the presence of CaHV-1 in our country [30].

Subsequently, this isolate was biologically characterized by (a) quantification of infectivity determining the viral titer, (b) determination of sensitivity to lipid solvent confirming the presence of viral envelope, (c) formation of eosinophilic intranuclear inclusion bodies that is characteristic of herpes viruses and (d) determination of the multiplication rate, verifying a kinetic behavior compatible with the one-step growth model [31].

2. Material and methods

This research was carried out in the Virology and Microbiology laboratories of the Department of Animal Preventive Medicine of the Faculty of Veterinary Sciences (FAVET) of the University of Chile.

2.1. Reactivation of the viral isolate RP5

2.1.1. Viral isolate

Viral inoculum of the RP5 isolate maintained at -20ºC in the laboratory of the Animal Virology Unit of the Faculty of Veterinary and Livestock Sciences of the University of Chile were used.

2.1.2. Cell cultures

Canine kidney cell cultures of the Madin-Darby Canine Kidney (MDCK) line were used, which were maintained in culture flasks (Easy Flask 75 ml, 25 cm^2) at 37ºC in Eagle's minimal essential medium (MEM) to which the antibiotics penicillin were added at a dose of 100 U/ml and streptomycin at a dose of 100 ug/ml, and supplemented with 10% bovine fetal serum (without CO_2).

2.1.3. Infection of cell cultures

MDCK cell cultures were infected with the viral isolate RP5 and they were kept at a temperature between 32º-33ºC, since this interval constitutes the optimum temperature for replication of this virus and the flasks were observed daily. culture (inoculated and uninfected controls) waiting to find the characteristic effect of rounding, cell lysis and cell detachment from the monolayer, for five days.

2.2. Partial detection of the CaHV-1 glycoprotein B (gB) gene by PCR

2.2.1. Obtaining viral DNA

Viral DNA was extracted from the cells of the MDCK line infected with the viral isolate RP5, once 80% destruction of the cell monolayer had been achieved, then 3 cycles of freezing/thawing of the inoculated flask were performed. in order to lyse the remaining cells. Subsequently, these were centrifuged at 1500xg for five minutes (Heraus Sepatech Biofuge®) and the supernatant was recovered to obtain the viral particles. A commercial DNA extraction and purification kit, Genomic DNA purification kit (Fermentas®) was used to obtain DNA. The technique consisted of mixing in Eppendorf tubes, with 200 µL of sample and 400 µL of the solution of lysis, which has the function of breaking the viral envelope, leaving the DNA free. The mixture was incubated at 65°C for five minutes, manually inverting the tubes

every one minute. Then, 600 µL of chloroform (Merk®) were added, gently mixed (inverting the tubes five times) and centrifuged at 10,000 rpm for two minutes (Heraus Sepatech Biofuge®).

In parallel, the precipitation solution was prepared by mixing 720 µL of nuclease-free water (Promega®) with 80 µL of the precipitation concentrate (10X) provided by the kit. Once centrifugation was complete, the sample was divided into two phases: the lower phase containing cell debris, and the upper phase containing viral DNA. The upper phase was transferred to a new Eppendorf tube and 800 µL of previously prepared precipitation solution was added, mixed gently at room temperature for two minutes, then centrifuged at 10,000 rpm for two minutes. During centrifugation, the DNA forms a pellet that remains in the tube, while the supernatant with impurities was completely removed. The pellet was dissolved with 100 µL of a 1.2 M NaCl solution provided by the kit, and its homogenization was ensured by using a tube shaker (vortex). This allowed the separation of DNA from protein and lipid debris. Then, 300 µL of cold ethanol (-20°C) were added and it was brought to -20°C for 10 minutes to achieve DNA precipitation. Subsequently, it was centrifuged at 10,000 rpm for four minutes and the supernatant was removed. Finally, a wash with 70% ethanol was performed and the purified DNA pellet was dissolved in 100 µL of nuclease-free water. This DNA was used immediately to carry out the PCR technique or it was stored at 4°C to be used within a week.

2.2.2. Polymerase Chain Reaction (PCR)

This method to amplify nucleic acids consists of three successive steps that together form a cycle:

Primers: two pairs of primers designed to amplify fragments of known size of 1286 bp [32] and 450 bp [33] of the gB gene were used, which were synthesized by the company Biosearch Technologies, Inc.

GN3	5`- TAA TTC ATA TGT CCC CTT TTT - 3`	
GN4	5`- GTC CTG TAT CTT CTA ACT CTG CT - 3`	Amplicon size 1286 bp
P1	5`- CCT AAA CCT ACT TCG GAT GA -3`	
P2	5`- GGC TTT AAA TGA ACT TCT CTG G -3`	Amplicon size 450 pb

2.3. Controls

DNA of *Salmonella* Enteritidis and cDNA of *Canine Distemper Virus* was used. As reagent control, water nuclease free (WNF) was used.

2.4. Reaction mixture

To achieve the purified DNA amplification mix, a commercial kit (2X PCR Master Mix; Fermentas®) was used, which contains the thermostable polymerase, deoxynucleotide triphosphates (dNTPs), the reaction buffer and $MgCl_2$. In the reaction mixture, 15 µL of 2X PCR Master Mix (DNA Taq Pol: 0.05 U/ µL; $MgCl_2$: 4 mM; DNTP's: 0.4 mM), 5 µL of each of the primers (0.1 nmol/ µL) and 5 µL of the DNA sample or template. A total volume of 30 µL was obtained, which was placed in 0.2 ml Eppendorf tubes. Homogenization was carried out using the vortex to ensure the mixture of the reagents.

2.5. DNA amplification

For the implementation of this technique, an Apollo thermocycler (CLP, USA) with 96 wells of 0.2 mL and a protocol that includes: GN3 and GN4 primers: initial denaturation at 95ºC for two minutes and then 35 cycles (denaturation at 95ºC for 60 seconds; alignment at 45ºC for 60 seconds; elongation at 72ºC for 180 seconds). Finally, an elongation stage at 72ºC for 10 minutes.

Primers P1 and P2: initial denaturation at 94ºC for three minutes and then 60 cycles (denaturation at 94ºC for 50 seconds; alignment at 49ºC for 50 seconds; extension at 72ºC for 50 seconds). Finally, an elongation stage at 72ºC for 10 minutes.

2.6. Visualization of amplified products

It was performed by 2% agarose gel electrophoresis (®) in Tris-borate buffer (90 mM Tris-borate, 10 mM EDTA) as solvent. The PCR product was mixed (6:1) with a commercial loading product (6X Mass Ruler Loading Dye Solution, Fermentas®). Electrophoresis was carried out at 100 V for 40 min. As molecular size marker, a standard containing DNA fragments between 100 and 2000 bp (Fermentas®) was used. After electrophoresis, the gel was immersed in ethidium bromide (0.5 µg/mL) (Fermelo®) and the bands were visualized in an ultraviolet light transilluminator (Transilluminator UVP®) and photographed.

2.7. Biosecurity measures

The laboratory work was carried out in accordance with the biosafety levels established for microbiology and animal virology laboratories, such as the use of clean material, correct disposal of waste, and the use of a closed apron and gloves in practical work. The visualization process of the amplified product involved the use of ethidium bromide and a UV light transilluminator. Due to this, an acrylic plate and glasses with a UV filter were used when viewing the gel. Subsequently, the elimination of the gel submerged in ethidium bromide contemplated its incineration, since the mentioned chemical compound has -among others- mutagenic properties.

2.8. Determination of the percentage of nucleotide identity with respect to GenBank®

2.8.1. Sequencing

The 450 bp amplified DNA fragment was sent to the sequencing center of the Genytec company, fulfilling its requirements.

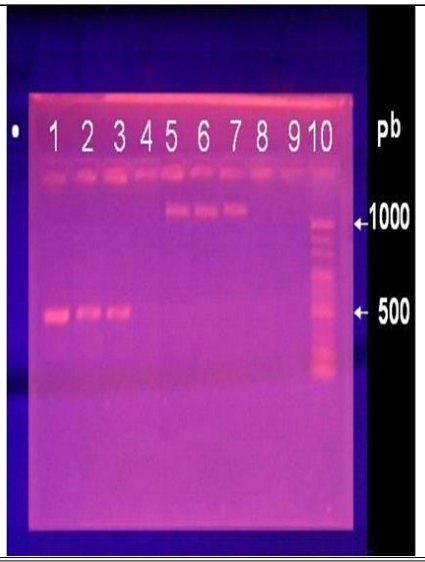
2.9. Analysis

The obtained sequences were aligned using the free access Clustal Ω program to obtain the consensus sequence. For de Nucleotide Identity Percentage the BLAST program was utilized.

3. Results

3.1. Partial detection of the CaHV-1 glycoprotein B (gB) gene by PCR

By performing the conventional PCR technique according to the protocols described, for the largest fragment equivalent to 1286 bp and for the smallest fragment equivalent to 450 bp, respectively, from the viral isolate RP5, it was possible to amplify the desired fragments of the gB gene. The bands obtained were located between 1200 and 1300 bp and between 400 and 500 bp, respectively, which were thick and sharp (Figure 1). A single band per lane was obtained, indicating that there was no non-specific amplification.



Lane 1 : Isolated RP5 DNA
Lane 2: Isolated RP5 DNA
Lane 3: RP5 Supernatant cell culture
Lane 4: Negative control 1 (DNA of *S.* Enteritidis)
Lane 5 : Isolated RP5 DNA
Lane 5 : Isolated RP5 DNA
Lane 7: Supernatant RP5 cell culture
Lane 8 : Negative control 2 (cDNA of CDV)
Lane 9: Reagent control (WNF)
Lane 10: MSM.
MSM: Molecular size marker (100-1000 bp).

Figure 1 Partial detection of the CaHV-1 glycoprotein B (gB) gene

3.2. Determination of the percentage of nucleotide identity with respect to GenBank®

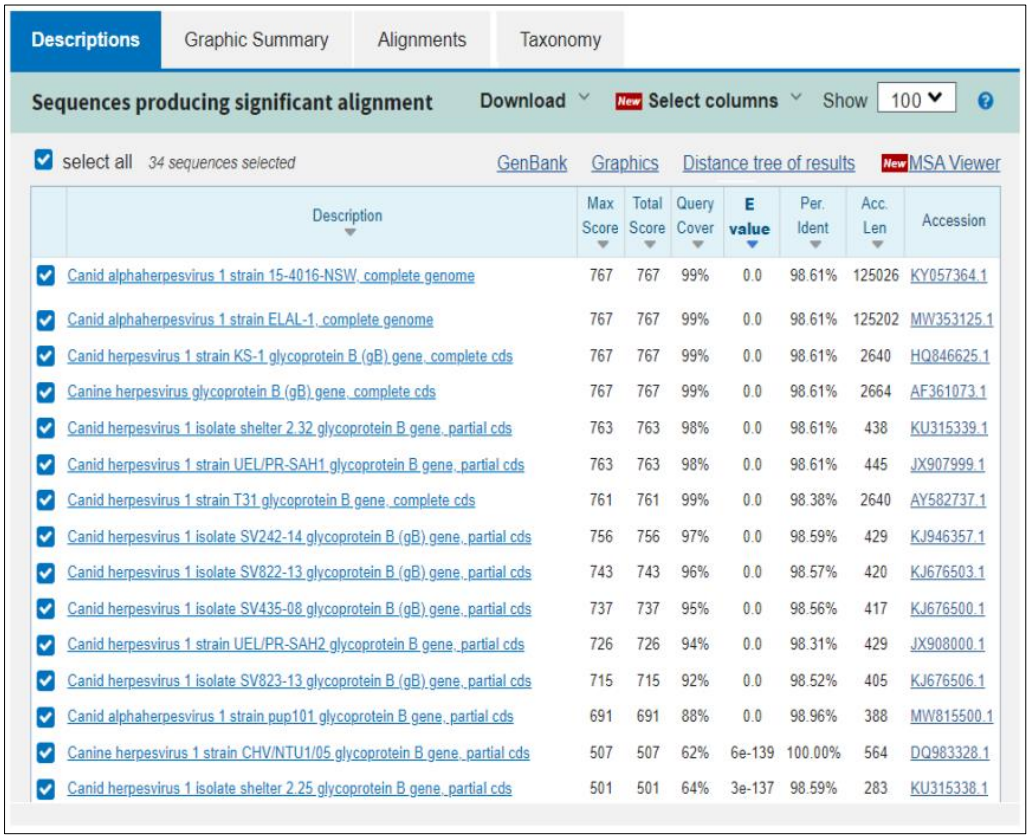
The sequences provided by Genytec Ltda. were aligned according to Clustal Ω and the LCM consensus sequence was obtained, which has a very high nucleotide identity percentage (NIP>98) with respect to official sequences (Genbank) according to BLAST software (Table 1).

3.3. Consensus sequence (LCM)

>LCM

AGCTTCGGATGATATTCGGGAAATTTTAGGTGAATCCCAAATTGAATCTGATGATACATCAACATTTTACAGGGACCCACCACA
ATCGGGATCAACATTGGTGCGTTTGGAGCCACCTAGAGCATGTCCTAACTATAAACTTGGTAAAAATTTTACAGAAGGAATTG
CTGTAATATTTAAGGAAAATATTTCTCCTTATAAATTTAAAGCTAATATATACTACAAAAATATTATTATCACCACTGTATGG
TCTGGAAGCACATATGCAGTAATTACTAATAGATATACAGATCGTGTACCTATAGGTGTTCCTGAAATTACAGAGTTGATTGA
TAGAAGAGGTATGTGTTTATCAAAAGCTGATTATATTCGTAATAATTATGAATTTACCGCATTTGATAAGGATGAAGACCC
CAGAGAAGTTCATTTAAAGCCCA

Table 1 Percentage of nucleotide identity of the LCM sequence



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignment — Download — New Select columns — Show 100

select all 34 sequences selected — GenBank — Graphics — Distance tree of results — New MSA Viewer

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Canid alphaherpesvirus 1 strain 15-4016-NSW, complete genome	767	767	99%	0.0	98.61%	125026	KY057364.1
Canid alphaherpesvirus 1 strain ELAL-1, complete genome	767	767	99%	0.0	98.61%	125202	MW353125.1
Canid herpesvirus 1 strain KS-1 glycoprotein B (gB) gene, complete cds	767	767	99%	0.0	98.61%	2640	HQ846625.1
Canine herpesvirus glycoprotein B (gB) gene, complete cds	767	767	99%	0.0	98.61%	2664	AF361073.1
Canid herpesvirus 1 isolate shelter 2.32 glycoprotein B gene, partial cds	763	763	98%	0.0	98.61%	438	KU315339.1
Canid herpesvirus 1 strain UEL/PR-SAH1 glycoprotein B gene, partial cds	763	763	98%	0.0	98.61%	445	JX907999.1
Canid herpesvirus 1 strain T31 glycoprotein B gene, complete cds	761	761	99%	0.0	98.38%	2640	AY582737.1
Canid herpesvirus 1 isolate SV242-14 glycoprotein B (gB) gene, partial cds	756	756	97%	0.0	98.59%	429	KJ946357.1
Canid herpesvirus 1 isolate SV822-13 glycoprotein B (gB) gene, partial cds	743	743	96%	0.0	98.57%	420	KJ676503.1
Canid herpesvirus 1 isolate SV435-08 glycoprotein B (gB) gene, partial cds	737	737	95%	0.0	98.56%	417	KJ676500.1
Canid herpesvirus 1 strain UEL/PR-SAH2 glycoprotein B gene, partial cds	726	726	94%	0.0	98.31%	429	JX908000.1
Canid herpesvirus 1 isolate SV823-13 glycoprotein B (gB) gene, partial cds	715	715	92%	0.0	98.52%	405	KJ676506.1
Canid alphaherpesvirus 1 strain pup101 glycoprotein B gene, partial cds	691	691	88%	0.0	98.96%	388	MW815500.1
Canine herpesvirus 1 strain CHV/NTU1/05 glycoprotein B gene, partial cds	507	507	62%	6e-139	100.00%	564	DQ983328.1
Canid herpesvirus 1 isolate shelter 2.25 glycoprotein B gene, partial cds	501	501	64%	3e-137	98.59%	283	KU315338.1

4. Discussion

CaHV-1 has been described in the United States of America as the cause of a fatal hemorrhagic disease in puppies less than four weeks old [1], a condition that has a great emotional impact for pet owners, as well as economic for pet sales companies.

In adults, although the presence of this virus was initially associated with the cause of infectious tracheobronchitis (ITB) or also called "kennel cough", over time it was found that it only corresponds to a secondary pathogen. However, the presentation of ITB may be an indication of the presence of CaHV-1. In a breeding stock, the most important are reproductive disorders and genital lesions caused by CaHV-1 [2, 13, 16].

The results obtained in this work using the PCR technique allow us to affirm that the RP5 isolate corresponds to CaHV-1, this will allow us to consider it as a national strain, and in the future to develop a vaccine to protect the presentation of the different cases in the canids, either as a pet or as a breeder.

The PCR technique is widely used worldwide for the detection of specific areas of a wide variety of pathogens. Its widespread use is due to its advantages: speed, in obtaining results compared to other diagnostic techniques; sensitivity, given by the ability to amplify sequences from a minimal amount of initial DNA, even from just one cell [34, 35,36] and specificity, given using *primers* [37].

A first consideration of the PCR technique is given by its sensitivity and in this regard one of the greatest precautions is the application of a standardized DNA extraction method, to obtain pure and undegraded DNA. Likewise, contamination of the samples under study must be avoided, contamination caused by external DNA present in the environment (aerosols), for which the use of latex gloves during the procedure is essential or by molecules from previous experiences, in which the importance will be in the sterilization process of the material to be used and of the cabinet in which the PCR reaction mixture is made. With this, it is possible to obtain reliable results, finally reflected in the absence of non-specific bands in the agarose gel [38, 39,40].

In this report, all the CaHV-1 samples subjected to PCR amplified fragments of expected size of 450 bp and 1286 bp, while in the negative control samples (*Salmonella* Enteritidis) subjected to PCR, no fragment was detected. The foregoing removes the possibility of a problem in the DNA extraction, which could have been reflected in obtaining false negatives. On the other hand, the samples of Salmonella Enteritidis, being negative, remove the possibility of obtaining false positives. Additionally, the obtaining of clear and unique bands in the case of positive samples, accounts for a specific amplification, product in addition to having taken all the pertinent measures of handling and sterility in the laboratory during the performance of this technique.

The technique implemented in this report corresponds to a second attempt to develop a diagnostic method for CaHV-1 in FAVET and this is only valid if there is support to determine whether the DNA fragments obtained certainly correspond to the sequences of the gB gene stored in GenBank for some years [8, 10, 41,42].

The function of the gB gene is important, hence it has been maintained evolutionarily and is part of the conserved genes in the genome of herpesviruses.

To determine the percentage of nucleotide identity between the amplified LCM and the gB gene described and stored in GenBank, the 450 bp fragment obtained and sequenced in this work was aligned using the Clustal Ω program obtaining 97% nucleotide identity according to BLAST. This result confirms that the national LCM amplification corresponds to a fragment of the glycoprotein B gene of CaHV-1.

This is of greater interest when considering that given the importance of the pathologies caused by CaHV-1 in pets, there are international laboratories that have included this viral diagnosis in their services using the PCR technique. Thus, diagnostic kits have even been created to be used in veterinary clinics that have the necessary infrastructure to carry out the technique. This is intended to make a diagnosis in the shortest time possible to take the necessary measures to avoid the death of puppies and the spread of the virus in a breeding stock due to a late diagnosis [43, 44].

Finally, currently in Chile there is no diagnostic technique for CaHV-1 based on molecular biology tools, there is only classical virology that involves viral isolation and some serological techniques. It should be noted that the only approximation to a molecular diagnostic technique was carried out in 2010 by means of a Titer Memory parallel to it, in which the CaHV-1 UL37 protein gene was detected by means of conventional PCR. In terms of sensitivity, the PCR protocol of this work would far exceed the one mentioned above [45].

5. Conclusion

The percentage of nucleotide identity (NIP>98) makes it possible to ensure that the RP5 isolate corresponds to CAHV-1, complements the molecular characterization initiated with the ul37 gene and its use as a diagnostic technique for CaHV-1 in Chile can be suggested.

Compliance with ethical standards

Acknowledgments

We want to thank all the students who have graduated with us and Dr. Aron Mosnaim of the Wolf Foundation, from Illinois, USA.

Disclosure of conflict of interest

There is no conflict of interest.

References

[1] Carmichael LE, Strandberg ID, Barnes FD. Identification of a cytopathogenic agent infectious for puppies as a canine herpes virus. Proc. Soc. Exp. Biol. Med. 1965; 120(3): 644-650.

[2] Carter GR, Wise DJ, Flores EF. Herpesviridae. In: A Concise Review of Veterinary Virology, G.R. Carter, D.J. Wise and E.F. Flores (Eds.) Publisher: International Veterinary Information Service (www.ivis.org), Ithaca, New York, USA. 2006.

[3] Geldard H, Geering W, Bagust T. Isolation of a herpesvirus from neonatal dogs in Australia. Aust. Vet. J. 1971; 47(6): 286-288.

[4] Hashimoto A, Hirai K, Miyoshi A, Shimakura S, Yagami K, Kato N, Kunihiro K, Fujiura A, Kitazawa K, Okada K, Fujimoto Y. Naturally occurring canine herpesvirus infection in Japan. [en línea]. Jpn. J. Vet. Sci. 1978; 40.

[5] Galosi C. Herpesvirus canino 1: Agente etiológico y enfermedad. Analecta Vet. 2007; 27(2): 28-35

[6] Gongora V, Sandoval N, Manchego A. Evidencia serológica de la presencia del virus herpes canino tipo 1 en la provincia de Lima. Rev. Investig. Vet. Peru. 2007; 18 (2): 154-157.

[7] ICTV. International Committee On Taxonomy Of Viruses. Virus Taxonomy. 2009.

[8] Davison A. Herpesvirus systematics. Vet. Microbiol. 2010; 143(1).

[9] Madigan MT, Martinko JM, Parker J. Brock. Biología de los microorganismos. 10ª ed. Pearson. Madrid, España. 2004; 1011.

[10] Murphy FA, Gibbs EPj, Horzinek MC, Studdert MJ. Veterinary virology. 3th ed. Academic Press. London, Inglaterra. 1999; 629.

[11] Lust G, Carmichael L. Properties of canine herpesvirus DNA. Proc. Soc. Exp. Biol. Med. 1974; 146(1): 213-217.

[12] Carmichael L, Green C. Infección por herpesvirus canino. In: Enfermedades infecciosas en perros y gatos. 2a ed. México. 2000; 31-36.

[13] Dumon C, Mimouni P. Mortinatalidad asociada a enfermedades infecciosas en la cría canina: brucelosis, herpesvirosis, micoplasmosis. In: Enciclopedia Veterinaria, Lafont A.; Durieux F. Ed. Elsevier. E-RE. 2005; 2250: 1-7.

[14] Schulze C, Baumgärtner W. Nested polymerase chain reaction and in situ hybridization for diagnosis of canine herpesvirus infection in puppies. Vet. Pathol. 1998; 35(3).

[15] Abbas A, Lichtman A, Pillai S. Inmunología celular y molecular. 6ª ed. Elsevier. Madrid, España. 2008; 577.

[16] Kawakami K, Ogawa H, Maeda K, Imai A, Ohashi E, Matsunaga S, Tohya Y, Ohshima T, Mochizuki M. Nosocomial outbreak of serious canine infectious tracheobronchitis (Kennel cough) caused by canine herpesvirus infection. J. Clin. Microbiol. 2010; 48(4): 1176-1181

[17] Ronsse V, Poulet H, Verstegen J, Thiry E. L'herpèsvirose canine. Ann. Mèd. Vèt. 2003; 147.

[18] Fenner F, Bachmann PA, Gibbs EPJ, Murphy FA, Studdert MJ, White DO. Virología veterinaria. Editorial Acribia. Zaragoza, España. 1992; 691

[19] Cabana E. Veterinary necropsy procedures. Veterinary Science & Medicine. Nueva Ecija, Philippines. 2008;44.

[20] Xuan X, Horimoto T, Limcumpao J, Tohya Y, Takahashi E, Mikami T. Glycoprotein-specific immune response in canine herpesvirus infection: Brief report. Arch. Virol. 1992; 122(3-4).

[21] Mullis K, Faloona F. Specific synthesis of DNA in vitro via a polymerase- catalysed chain reaction. Meth. Enzymol. 1987; 155: 335–350.

[22] Burgesser K, Hotaling S, Schiebel A, Ashbaugh S, Roberts S, Collins J. Comparison of PCR, virus isolation, and indirect fluorescent antibody staining in the detection of naturally occurring feline herpesvirus infections. [en línea] J. Vet. Diagn. Invest. 1999; 11(2).

[23] Reubel G, Pekin J, Wright J, Venables D, French N. Corticosteroid treatment does nor reactivate canine herpesvirus in red foxes. J. Wildl. Dis. 2004; 40(2): 238-248.

[24] Green C, Watson A. Fármacos antivirales. In: Enfermedades infecciosas en perros y gatos. 2a ed. México. 2000; 7-10.

[25] Limbach K, Limbach P, Conte D, Paoletti, E. Nucleotide sequence of the genes encoding the canine herpesvirus gB, gC and gD homologues. J. Gen. Virol. 1994; 75(8).

[26] Babiuk L, L'italien J, Van Drunen Littel-Van Den Hurk S, Zamb T, Lawman M, Hughes G, Gifford G. Protection of cattle from bovine herpesvirus type 1 (BHV-1) infection by immunization with individual viral glycoproteins. Virology. 1987; 159(1): 57-66.

[27] Mettenleiter TC, Spear PG. Glycoprotein gB (gII) of pseudorabies virus can functionally substitute for glycoprotein gB in herpes simplex virus type 1. J. Virol. 1994; 68(1).

[28] NCBI. The National Center For Biotechnology Information. Canine herpesvirus glycoprotein B (gB) gene, complete cds. 2021.

[29] Larenas J, Santibáñez MC, Berríos P. Primeros antecedentes en Chile de infección por herpes canino con mortalidad neonatal. Mevepa. 1992; 6(1): 13-16.

[30] Navarro C, Celedón M, Pizarro J. Detección de virus herpes canino tipo 1 en Chile. Arch. Med. Vet. 2003; 35(2): 227-231.

[31] Navarro C, Celedón M, Pizarro J, Gaggero A. Virus herpes canino en Chile: Propiedades biológicas. Arch. Med. Vet. 2005; 37(2): 133-137.

[32] Erles K, Dubovi E, Brooks H, Brownlie J. Longitudinal Study of Viruses Associated with Canine Infectious Respiratory Disease. J. Clin. Microbiol. 2004; 42(10): 4524–4529.

[33] Ronsse V, Verstegen J, Onclin K, Farnir F, Poulet H. Risk factors and reproductive disorders associated withcanine herpesvirus-1 (CHV-1). Theriogenology. 2004; 61(4): 619–636.

[34] Gibbs RA. DNA Amplification by the Polymerase Chain Reaction. Anal. Chem. 1990; 62(13).

[35] Erlich HA, Gelfand D, Sninsky JJ. Recent Advances in the Polymerase Chain Reaction. Science. 1991; 252(5013): 1643-1651.

[36] Arnheim N, Erlich H. Polymerase Chain Reaction Strategy. Annu. Rev. Biochem. 1992; 61: 131-156.

[37] Belák S, Ballagi-Pordány A. Application of the polymerase chain reaction (PCR) in veterinary diagnostic virology. Vet Res Commun. 1993; 17(1): 55-72.

[38] Dieffenbach C, Dveksler G. Setting up a PCR laboratory. Genome Res. 1993; 3.

[39] Torres AG, Baca BE. Reacción en Cadena de la Polimerasa. Elementos. 1995; 23(3): 16-21.

[40] Mifflin TE. Setting Up a PCR Laboratory. In: Dieffenbach, C.; Dveksler, G. (Eds.). PCR Primer: a laboratory manual. 2th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. New York, USA. 2003; 5-14.

[41] McGeoch DJ, Cook S. Molecular phylogeny of the Alphaherpesvirinae subfamily and a proposed evolutionary timescale. J. Mol. Biol. 1994; 238(1): 9–22.

[42] McGeoch DJ, Cook S, Dolan A, Jamieson FE, Telford EAR. Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. J. Mol. Biol. 1995; 247(3): 443–458.

[43] Genekam Biotechnology AG. Ready to use PCR kits (DNA viruses). 2007.

[44] Laboratorio D'analisi Veterinarie San Marco. Biología molecular para enfermedades infecciosas. 2008.

[45] Fuentes A, Jara MA, Navarro. Detection of ul37 gen in Canine Herpes Virus by means Polymerase Chain Reaction. In arbitration. 2021.