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Detection of ul37 gene from *Canine Herpes Virus* by polymerase chain reaction

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Abstract
In Chile, the *Canine Herpes Virus* has been detected by classical virology techniques such as isolation in cell cultures and subsequent cytolysis of affected cells; by immunofluorescence; by the presence of inclusion bodies or by viral kinetic studies which has made it possible to complete the biological analysis of a national isolate called RP5. In contrast, in the present work, the molecular detection of the ul37 gene of CaHV-1 was carried out, a gene that codes for the viral UL37 protein.
The visualization of the amplicon was carried out by electrophoresis in agarose gel at 2% at 90 volts for 90 minutes. After electrophoresis, the gel was incubated in ethidium bromide, subsequently washed, placed in an ultraviolet light transilluminator, and finally photographed. All the cell culture supernatants used were positive for the described PCR, observing a band of around 500 bp, which agrees with what is described in the literature.
The amplified product was purified, sequenced and the percentage of nucleotide identity (PNI) was determined by BLAST program. Thus, it was determined an NIP>97 and strongly suggests that the sequence obtained it belongs to CaHV-1.
Thus, we can conclude that this methodology allows the detection of a DNA amplicon with characteristics compatible with those described for the CaHV-1, and the ul37 gene as target using a molecular tool that meets the characteristics of current diagnosis: fast, sensitive, specific and low cost.
This method undoubtedly contributes to solving an existing problem in canine breeding farms, by having the possibility of offering the detection of CaHV-1 in small animals as companion pets, an area of sustained growth.

Keywords: CaHV-1, PCR, molecular diagnosis, ul37 gen

Introduction
Canine Herpes Virus type 1 (CaHV-1) was first described in 1965 by three different groups of scientists and has been considered a rare clinical agent for many years (Ronsse *et al*., 2003; Bottinelli *et al*., 2016) [46, 5].
Although in the mid-1960s it was described as a virus that causes infections of the external mucosa of the urogenital apparatus in adult dogs, forming part of the complex of pathogens involved in the so-called "kennel cough", it was also associated with to fatal hemorrhagic disease in puppies less than 4 weeks old (Carmichael *et al*., 1965) [11].
In the following years, studies began to determine its true pathogenicity, with the first experimental infection being carried out in pathogen-free dogs between 5 and 12 weeks of age. In addition, the first work was carried out to know its genome, comparing its size with that of 3 other herpes viruses. At the same time, the first viral isolation was carried out in the reproductive tract of female dogs with reproductive problems (Poste and King, 1971; Appel and Bemis, 1978; Bottinelli *et al*., 2016) [42, 1, 5].
Currently, the presence of the virus is worldwide and recent studies suggest a high seroprevalence in the canine population the virus has been isolated in many countries and recent studies in Europe suggest that it is an enzootic agent in the domestic dog population (Lacheretz and Cognard, 1998; Ronsse *et al*., 2003; De Palma, 2010) [29, 46, 10].

Recent serological studies indicate a high level of infection in the canine population and in general, it is estimated that more than 40% of dogs in Europe had contact with the virus (Buonavoglia and Martella, 2007) [7]. In a study carried out in the USA, they investigated canine respiratory disease, finding that 6% of dogs had a significant amount of antibodies against CaHV-1, during the clinical phase of the disease (Morresey, 2004) [36]. The prevalence of antibodies varies between different countries in ranges from 9 to 88% (Buonavoglia and Martella 2007) [7].

One developed vaccine was successfully tested in 2001, where six CaHV-1-free bitches were vaccinated against the virus ten days after mating. At 3 days after delivery, all pups exposed to the virus survived, unlike a control group (Poulet *et al*., 2001; Larsen *et al*., 2015) [43, 31].

Taxonomic classification

CaHV-1 is a virus belonging to the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. The Order *Herpesvirales* also includes other herpesviruses that infect hosts as diverse as mammals, reptiles and birds, belonging to two other families: *Alloherpesviridae*, which infect fish and amphibians, and *Malacoherpesviridae*, with a single member that infect bivalve molluscs (Davison, 2010) [14].

The *Herpesviridae* family includes 3 already accepted subfamilies: *Alphaherpesviridae*, *Betaherpesviridae* and *Gamaherpesviridae*, with a still unclassified subfamily and unclassified herpes viruses. Thus, more than 130 different species of herpesviruses have been described, with an impressive range of affected species. These viruses have a genome composed of linear, double-stranded DNA and a capsid with icosahedral symmetry made up of 162 capsomeres. The diameter of the virion is 180 to 200 nm. Surrounding the capsid is the integument, an amorphous matrix containing numerous proteins with enzymatic activity (Plummer *et al*., 1996; Remond *et al*., 1996; Boehmer and Lehman, 1997; Louten, 2016) [41, 44, 4].

Biological Characteristics

The presence of an envelope made up of glycoproteins and lipoproteins gives the virion a high sensitivity to lipid solvents and common disinfectants. It has a "half-life" of 5 hours at 37ºC. It is unstable under both acidic (pH <5) and alkaline (pH >8) conditions. Its optimum multiplication temperature is 32-33ºC, with a decrease in viral yield at 36ºC and it is inactivated at temperatures above 40ºC. Its infective capacity begins to decrease after 24 hours at 4ºC (Carmichael *et al*., 1998) [10].

Compared to other *Alphaherpesviruses*, CaHV-1 has a narrow range of host cells. It only multiplies in primary cultures of cells of canine origin such as kidney and testis or cell lines such as Madin Darby Canine Kidney (MDCK). In these cell lines, the virus produces a cytopathic effect (CPE) characterized by the presence of lytic foci, rounding of the infected cells with increased refringence, formation of syncytia and subsequent total detachment of the monolayer (Love and Hurxtable, 1976; Murphy *et al*., 1999; Kim *et al*., 2005) [33, 38, 28]. Another important characteristic of herpesviruses is their ability to enter a latency state, that is, it is a state in which no viral progeny is produced, which occurs mainly in nerve ganglia (Boehmer and Lehman, 1997; Grinde, 2013; Brown, 2017) [4, 21, 6].

Hosts

Although CaHV-1 affects domestic dogs, both puppies and adults, a virus like CaHV-1 was isolated from captive coyote pups (Robinson *et al*., 2005; Buonavoglia and Martella., 2007) [45, 7]. On the other hand, the detection of antibodies has been described in river otters (*Lontra canadiensis*) in North America as well as in red foxes (Ronsse *et al*., 2003; Bottinelli *et al*., 2016) [46, 5].

Clinical Signology

In adult dogs, the virus causes only a mild upper respiratory tract or genital infection (Hill and Maré, 1974) [24]. However, in the pregnant bitch, infection can induce stillbirth, abortion, and mortality among offspring (Hashimoto *et al*., 1982) [23]. A notable feature of all *Alphaherpesviruses* is the ability to induce latent infection in the trigeminal nerve and ganglia of multiple tissues Animals that survive infection remain asymptomatic carriers for life. Most infections in adults are subclinical (Carmichael, 1970; Hashimoto and Hirai, 1986; Burr *et al*., 1996; Verzosa *et al*., 2021) [9, 22, 8, 53].

Disease in puppies

The death of puppies between 1 and 4 weeks of age is common. Puppies rarely die if they are 2-3 weeks old at the time of exposure. The duration of the disease in newborn puppies is 1 to 3 days. Signs include anorexia, dyspnea, abdominal tenderness, incoordination, and often loose, yellow-green stools. There may be a serous, or hemorrhagic, nasal discharge. Petechiae are common on mucous membranes. Rectal temperature is not elevated. Thrombocytopenia has been reported in moribund puppies (Carmichael and Green, 1998) [10]. Puppies exposed to canine herpes virus, either in utero, during birth, or within the first 2 weeks of life, can develop meningoencephalitis that may preferentially affect the cerebellum, and almost all puppies with an active infection early in life succumb to infection. However, some puppies survive with residual CNS lesions. These puppies may be affected with retinal dysplasia, as this tissue is undergoing active differentiation at the time of infection, which would affect its conformation (Evermann *et al*., 2011) [27].

Genital disease in adults

The genital form of the disease in females is characterized by vaginal hyperemia and lymphoid hyperplasia. The occasional appearance of papules or vesicular lesions in the genital mucosa evolve in 15 to 30 days with ulceration, and may return in the following proestrus, presenting hyperplasia of the submucosal glands. In males it causes papules on the penis. The lesions seen in the vagina, penis, and foreskin are generally self-limited (Galosi, 2007) [19].

Reproductive problems

Clinical signs in puppies depend on age, presence of maternal antibodies, stress, and concurrent infections (Hashimoto and Hirai, 1986; Kraft *et al*., 1986) [22, 27]. If the infection occurs during pregnancy, miscarriages or sudden perinatal death occur within the first 48 hours of birth. Fetal death, mummification, abortions, premature or abnormal births could be associated with prenatal infection (Hashimoto, 1986) [22]. Females giving birth to infected litters develop immunity for the next oestrus and, with rare exceptions, subsequent litters are normal (Hirai *et al*., 1978) [25].

Respiratory signs

The participation of CaHV-1 in canine infectious tracheobronchitis (CIT), also known as "kennel cough", which is an infectious-contagious disease that is frequently observed in places where there is a large concentration of animals such as farms, training centers, is described and kennels (Erles *et al*., 2004; Buonavoglia and Martella, 2007) [8, 7]. This infection is caused by several other etiological agents that can act alone or in association with each other: *Bordetella bronchiseptica*, *Mycoplasma sp*, *Canine Adenovirus* type 2 (AVC-2), *Paramyxovirus* type 2 (Appel and Bemis, 1978; McCandlish *et al*., 1978; Erles and Brownlie, 2005) [1, 17]. CaHV-1 participates in CIT, that is, its importance as an etiological agent in this clinical signology is not entirely clear determined (Erles *et al*., 2004) [8]. The initial picture can be maintained for months presenting problems in the respiratory system (Bemis *et al*., 1977; Ueland, 2003) [3, 52].

Prevention

Prevention consists of providing warmth to the pups by all possible means to maintain their rectal temperature above 37ºC. CaHV-1 has little resistance in the external environment and is therefore effectively destroyed by most disinfectants (sodium hypochlorite, formalin, quaternary ammonium salts). In addition, it is very sensitive to heat (>37ºC), but very resistant to cold. Animals carrying the virus should be separated from others to avoid any direct mucosal-to-mucosal (genital or respiratory) contact with healthy animals (Galosi, 2007; Kapil, 2015) [19, 26].

Vaccination

Vaccination of the bitch at oestrus and around fifty days after mating could confer protection against the acute neonatal form (Thébault, 2004) [51]. To counteract canine herpes virus infection, medical prophylaxis is used using the inactivated EURICAN Herpes205 ® vaccine (Merial Laboratory), a vaccine that has been created specifically in the Bizerte Spain canine center. Its efficacy was evaluated in pregnant females by measuring the fetal death rate of the pups, as well as the analysis of pathological lesions observed in the pups (Chabchoub *et al*., 2006) [12].

The UL37 protein

A combination of genetic, biochemical, and protein analyzes of *Alphaherpesvirus* suggest that the integument contains around 20 viral proteins, such as; UL11, UL13, UL14, UL21, VP1-3, UL37, UL41, UL46, UL47, UL48, UL49, UL51, ICP0, ICP4, US3, US10 and US11. These proteins are arranged interacting with each other forming an intricate complex that joins the capsid with the intrategument domains of the envelope glycoproteins (Mettenleiter, 2003) [35]. One of these proteins is the UL37 protein, a protein conserved among the members of the *Herpesviridae* family, of late type and described as an essential protein for viral replication, participating in the egress of the capsid from the cell nucleus and in the acquisition of the viral envelope (Loret *et al*., 2008) [32].

The ul37 gene encodes a phosphorylated 120-kDa polypeptide that is expressed late in the virus replication cycle. In immunofluorescence assays performed on cells infected with Herpes Simplex virus, it has been described that the UL37 polypeptide is distributed throughout the infected cell, predominantly in the cytoplasm (Schmitz *et al*., 1995) [49].

In detail, the functions of these proteins are related to:

a) Regulate the expression of viral and host cell genes at different stages of infection (Batterson and Rotzman, 1983)
b) Participate in the transport of the capsid through the cell (Sodeik *et al*., 1997).
c) Intervene in the insertion of viral DNA in the cell nucleus (Batterson and Rotzman, 1983)
d) Contribute to the encapsulation of the viral DNA of the progeny (Salmon *et al*., 1998)
e) Participate in the exit of the virion from the host cell (Desai, 2000)

National situation of the *Canine Herpes Virus*

At the national level, hemorrhagic disease in puppies under 4 weeks of age and the associated virus were initially suggested in 1992, where an equine fetal kidney cell line was used to study the virus in the laboratory. Its presence was suggested by the presentation of perinatal death in puppies that showed the presence of petechiae in the kidney and liver and many eosinophilic intranuclear inclusion bodies.

The infection of cultures of the equine kidney cell line presented the characteristic cytopathic effect of herpes virus: cell lysis (Larenas *et al*., 1992) [30]. This article constituted the first documented national information regarding the presence of CaHV-1 in Chile.

Subsequently, the detection of the virus was reported by obtaining a viral isolate called RP5, which produces the characteristic cytopathic effect of herpes viruses: cell lysis and that shows high reactivity in a direct immunofluorescence test that uses a polyclonal antibody conjugated with fluorescein isothiocyanate, directed against CaHV-1 envelope glycoproteins (Navarro *et al*., 2003) [39].

Material and Methods

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

Detection of the UL37 protein gene in CaHV-1 by conventional PCR

Obtaining viral DNA. Viral DNA was extracted from MDCK cells infected with the RP5 isolate, once 80% cell monolayer destruction had been achieved. Three freeze/thaw cycles were performed on the inoculated flask, to lyse the remaining cells. They were subjected to centrifugation at 1,500xg for 5 minutes and the supernatant was recovered to obtain the viral particles. Chomczynski's solution (Winkler, Ltda.) was used to obtain DNA. This technique consisted of mixing 200 ul of sample with 1 ml of reagent and inverting the sample 3 times. 0.5 ml of absolute ethanol were added, followed by centrifugation at 5,000×g for 5 minutes. The precipitate was washed 2 times with 1 ml of 75% ethanol, inverting the tube 4 times and centrifuged at 1,000xg for 2 minutes. The supernatant was removed and the DNA was solubilized by adding 0.2 ml of nuclease-free water (Chomczynski *et al*., 1997) [13].

Conventional PCR. To perform this technique, an Apollo Thermocycler (CLP, USA) with 96 wells (0.2 mL) was used. Primers. For the detection of canine herpes virus (CaHV-1), primers were used: RP11-CHV1: 5'-

AAGAGCTCGTGTTAGTGAAAAT-3´ and RP22-CHV2: 5'-TAAACCCGCTGGATGATAC-3', that were designed to amplify a 494 base pair (bp) fragment of a region homologous to the ul37 protein gene of the human herpes simplex virus (Erles *et al*., 2004) [18].

Reaction mixture. In a 0.2 ml Eppendorf tube, 5 ul of viral DNA, 15 ul of 2X PCR Master Mix (recombinant Taq DNA polymerase, $MgCL_2$ and dNTPs) and 5 ul of each primer were mixed. Bacterial DNA from *Salmonella* Enteritidis and cDNA of *Canine distemper Virus* (CDV) was used as a negative control. Water nuclease free (WNF) was used as reagent reactive control.

DNA amplification. The PCR protocol includes an initial denaturation at 95°C for 1 minute, followed by 35 cycles of PCR (denaturation 95°C, 1 minute; annealing 49°C, 40 seconds; elongation 72°C, 1 minute). Finally, a final elongation stage at 72°C for 10 minutes. The expected size of the PCR amplification product is 494 bp (Erles *et al*.; 2004) [18]

Visualization of the amplified product by PCR

The amplified DNA was visualized by 2% agarose gel electrophoresis (Winkler®) in Tris acetate EDTA buffer (TAE, Fermentas ®). The PCR product was mixed (5:1) with a loading buffer (6X Mass Ruler Loading Dye Solution, Fermentas ®). Electrophoresis was carried out at 90 volts for 90 minutes. As molecular size marker, a standard containing DNA fragments between 100 and 1000 bp (Fermentas ®) was used. After electrophoresis, the gel was incubated in ethidium bromide (Fermelo ®) for 20 minutes and the DNA was visualized in an ultraviolet light transilluminator (Transilluminator UVP ®).

Biosafety Measures

The laboratory work was carried out in accordance with the biosafety levels established for microbiology and animal virology laboratories: use of clean material, correct disposal of waste, and the use of a closed apron and gloves. 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 visualizing the gel. Subsequently, the elimination of the gel incubated in ethidium bromide was eliminated according to the waste management protocol established in the FAVET, which contemplated its incineration, since the chemical compound mentioned has mutagenic properties.

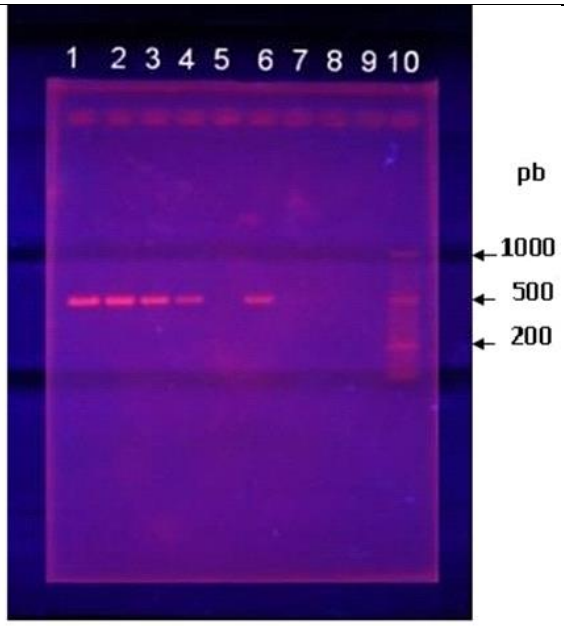
Identification of DNA amplified by PCR. Sequencing. The DNA fragments were sent in triplicate to the Sequencing Center of the GENYTEC company according to their requirements. The sequences were made using the Big Dye Terminator Kit, from Applied Biosystems, and the ABI PRISM 310 Genetic Analyzer equipment (GENYTEC specifications) was used for their reading.

Analysis. Using the freely accessible online program called Clustal Ω. The sequences obtained were aligned to obtain the consensus sequence and the percentage of nucleotide identity was established by BLAST program.

Results

Detection of the canine herpesvirus UL37 protein gene in the RP5 isolate by PCR.

When performing the PCR according to the established protocol (Erles *et al*., 2004) [18], it was possible to obtain a DNA fragment with a molecular size of around 500 base pairs (bp). The amplified band turned out to be unique (Figure 2), without nonspecific amplifications.



Lane	Content
1	DNA of RP5
2	DNA of RP5
3	RP5 supernatant cell culture
4	RP5 supernatant cell culture
5	Negative control: DNAc CDV
6	DNA of RP5
7	Negative control: DNAc CDV
8	Reagent Control: WNF
9	Negative control: DNA S. Enteritidis
10	MSM

MSM: Molecular Size Marker (100-1000 bp); Fermentas®

Fig 2: Detection of the canine herpesvirus UL37 protein gene in the RP5 isolate

Sequencing. Determination of nucleotide identity

The amplified obtained were successfully sequenced. A multiple alignment was carried out by Clustal Ω to obtain the consensus sequence (AFA) and thus compare it with the official GenBank and obtained an NIP>97% (see Annex 1)

Consensus sequence (AFA)

TGGGAAACGATACAGTCCAGTACAACCCCATTTCA
AGTTATAGATGCGTTAATTGGAGCTGGTTTTACACC
TATTCATTGTGATATTTTAGAAAATGTTATTGTAGA
TCAATATTCAAAAATTAAAAATGGAAATCTATCTT

TAGATGAAAAAAATTCGCTAAACGATATTCAACAA
ATTATAGGATGTATATCAATTGTTGGTGGTTTGATA
TTTAAACTCTTACGAAAGTATGGTTATGGTTTAGAA
TATATAAAATTTTATACATCAACCCTTTCTGATTTA
GAAGCTATATATGGTGAATTATTAAATTCAATTGG
ATTACCACATGGGGGTGTTGAGCAAACAATTAGAC
ATTGTATTGCTCCAATAGCCACTTCTTCCCGTTATA
GAATCAACTTATACATTGTTATTTCTTCCATGGTTT
ATT

Discussion

CaHV-1 is one of the main infectious agents causing reproductive disorders in canines and one of the etiological agents that occasionally participates in the respiratory syndrome called infectious tracheobronchitis or "kennel cough", although its role in this last pathology for many years it remained controversial (Buonavoglia and Martella, 2007) [7]. In addition, it has been described as the cause of a neonatal disease that was first described in the United States (Carmichael *et al*., 1965) [11] and currently numerous reports give it a worldwide distribution. In Chile, a native strain called RP5 has been isolated and biologically characterized (Navarro *et al*., 2005) [40].

Currently, in Chile there are no diagnostic methods for the detection of CaHV-1 in clinical cases suspected of being infected with this virus. This report is a first step in the implementation of a precise, sensitive and effective technique for the diagnosis of suspected cases of canine herpes virus.

The *Polymerase Chain Reaction* (PCR) technique is today one of the fastest methodologies for the detection of pathogens. This technique has a high sensitivity and specificity, presenting the possibility of analyzing many samples at the same time (Mullis, 1990; Schrank *et al*., 2001) [37, 48]. For this reason, this technique is increasingly used as a diagnostic technique in human medicine, in pathologies as diverse as the outbreak of H1N1 influenza a year ago, cases that were diagnosed using the PCR technique, as a diagnostic method for suspicious cases. In medicine, the PCR technique is used because it is a diagnostic technique that detects the target gene in different types of samples: feces, cultures and biological samples.

The objective of this work was to implement the molecular diagnosis of the CaHV-1 by means of the PCR technique, to complement the biological characterization of the RP5 isolate (Navarro *et al*., 2005) [40]. In relation to the work proposed and developed in this work, it would be interesting to note some considerations:

First, the laboratory work was carried out without major inconveniences and with the experience obtained it was corroborated that the most critical moment of the PCR test is the mixing of the PCR reaction. Not only because of the volumes of reagents used in the reaction mixture, but also because any contamination at this stage will cause errors in the amplification, which translates into erroneous, or at least confusing, results. To address these drawbacks, it is recommended to rigorously follow laboratory practices, such as using calibrated pipettes, working instruments in good condition, avoiding the presence of dust or any contamination, and using reagents of certified quality guaranteed by the supplier (Wolcott, 1992) [54]. Therefore, when performing the PCR tests in the laboratory, precautionary measures were taken to avoid errors in the amplification, using a closed and exclusive sector to mix the PCR reagents, a sector that was subjected to ultraviolet light the night before use, in order to prevent the samples from becoming contaminated with foreign DNA or aerosols present in the laboratory.

Second, performing a conventional PCR using primers that amplify a 494 base pair fragment of a region homologous to the UL37 protein gene of human HSV-1 (Erles *et al*., 2004) [18] allowed obtaining a fragment of DNA of approximately 500 bp, clear, unique and that would presumably correspond to the target sequence of the herpesvirus UL37 protein gene. This, would support that the choice of the ul37 gene as a target to be detected by PCR, represented a valid and valuable alternative to start the molecular characterization of the native RP5 isolate.

Thirdly, if the specificity of a PCR reaction is given by the primers used, the implemented technique complies with this characteristic, since when using DNA samples of *Salmonella* Enteritidis strains (negative control) there was no amplification, corroborated by the absence of fragments or fluorescent bands in the visualization of the agarose gel. Therefore, the primers used are specific for *Alphaherpesviruses*.

Fourth, this PCR technique proves to be an effective technique for the diagnosis of CaHV-1, over other diagnostic tests such as viral isolation or serology, having the advantage of the high sensitivity of the technique, in addition to the advantage in delivery times of results (approximately 5 hours). This is highly important from the economic point of view because this disease causes reproductive problems, which makes it necessary to detect CaHV-1 early to rule out possible candidates in the choice of breeders.

Fifth, the analysis of the sequences obtained allows us to affirm that the viral isolate RP5 represents a valid alternative to be used as a positive control, since the comparison of the nucleotide identity percentage (NIP) between the amplified obtained (AFA sequence) and the sequences of the ul37 gene present in GenBank. In other words, these results allow us to establish that the PCR technique described allows us to detect a gene present in the RP5 isolate and that the sequence of this gene has very high nucleotide identity (NIP>97%) with the sequence described for the CaHV-1 glycoprotein ul37 gene, according to official information in GenBank.

Finally, the detection of CaHV-1 in samples of suspected animals is the real challenge for the future and the final purpose of the investigation, since in Chile there is no diagnostic technique for this virus yet.

Conclusion

This report describes for the first time in Chile the molecular diagnosis of the canine herpes virus by detecting the ul37 gene using the PCR technique and the percentage of nucleotide identity of 94% allows us to affirm that the national isolate RP5 corresponds to CAHV-1. Therefore, this molecular characterization complements the previous biological characterization.

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