

Use of Multiplex polymerase chain reaction in Detection of canine her pesvirusglycoproteinb Gene

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Abstract: *Viruses will always need the metabolic machinery of the infected cell to replicate its genetic material and produce multiple copies of the original virus. In this process, some viruses have the capacity to produce a cytopathic event called cellular lysis. Among the viruses that produce this effect we can find Herpes virus, described in insects, reptiles, amphibians, and mollusks; as well as almost all bird and mammal species ever investigated. One of the virus of veterinary interest is Canine Herpes Virus Type 1 (CaHV1), due to its persistence in canine kennels it creates considerable economic losses, that can produce the shutdown of these kinds of businesses. CaHV1 causes a high number of clinical cases, especially in puppies younger than four weeks old, where a mortality rate of 90% has been described. Even though in Chile its detection and biological characterization was made a few years ago, obtaining a native isolate named RP5, a quick detection method does not exist. Due to this, the objective of this study was the simultaneous detection of two fragments of the CaHV1 glycoprotein B gene in the same PCR (multiplex variant) from the RP5 isolate. Thus, by establishing the optimal times for elongation and alignment, a tool of molecular diagnostic was implemented, and could be used and compared with other PCR protocols that are currently being developed in other thesis in the Virology Laboratory of our Faculty.*

Keywords: *canine herpes virus, multiplex PCR, molecular diagnostic.*

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I. Introduction

Canine Herpes Virus Type 1 (CaHV1) was first described in the United States as the causative agent of a fatal septicemic disease in puppies under 4 weeks of age (Carmichael et al., 1965; Gadsden et al., 2012). In adult dogs causes infection of the external mucosa of the genital tract causing, among other symptoms, a mild vesicular vaginitis; It is also one of the causes of the respiratory syndrome called "cough of the kennels", which corresponds to a tracheobronchitis (Carter et al., 2006; Kawakami et al., 2010). CaHV1 belong the Herpesvirales order, Herpesviridae family, Alphaherpesvirinae subfamily, Varicellovirus genus (ICTV, 2018). The Herpesviridae family is composed of many viruses that act as infectious agents in various diseases, both in humans and animals (Madigan et al., 2003) and it is made up of four subfamilies: Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae and a fourth that has not yet been named (ICTV, 2018). The division into subfamilies was initially due to observed differences in the biological characteristics of its members. However, molecular antecedents that include genomic sequence and phylogenetic analysis have now been considered (Murphy et al., 1999; ICTV, 2018). The Alphaherpesvirinae subfamily belong to rapidly propagating virions that perform lytic cycles in infected cells, possess a wide host range and produce latent infections, especially in the sensory ganglia (Boehmer and Lehman, 1997). This phenomenon of latency in the host can persist for long periods of time and then be activated, for example under stress conditions (Madigan et al., 2003). Five genera have been described: Iltovirus, Mardivirus, Simplex virus, Varicellovirus and a fifth that still does not receive a name (ICTV, 2018). It should be noted that most of the herpesviruses with veterinary importance belong to the genus Varicellovirus (Carter et al., 2006). Herpes viruses have a DNA genome, whose linear double-stranded molecule reaches a size that varies between 125 and 235 thousand base pairs (bp). This genome is surrounded by a capsid of icosahedral symmetry of approximately 125nm. Surrounding the capsid, there is a protein fibrous structure called the tegument and a lipid envelope with projections distributed on the surface. The final size of the viral particle varies between 120 and 300 nm (Madigan et al., 2003; Carter et al., 2006). The high fragility of the virion is directly related to the presence of a glycoprotein and lipid envelope, which can be easily destroyed by heat, ultraviolet rays, lipid solvents and common disinfectants (Murphy et al., 1999). The multiplication of the virion is optimal between 32 and 33°C, at that temperature a rapid replication is appreciated with lysis of the infected cells, however, it is easily inactivated at temperatures above 40° C (Dumon and Mimouni, 2005). The replication of a Herpesvirus involves a process consisting of 5 stages:

adsorption, penetration, synthesis of nucleic acid and proteins, assembly and finally release (lysis) (Murphy et al., 1999; Madigan et al., 2003). The adsorption is highly specific; at this stage the virus proteins interact with cell receptors, which will determine which cells will be susceptible to being infected. For replication to be effective, new copies of the viral genome must be synthesized, in addition to the synthesis of virus-specific proteins. Therefore, fixation and even penetration into a susceptible cell does not guarantee viral multiplication (Madigan et al., 2003). During replication, three classes of mRNA can be generated: the immediate early (α) that encodes five regulatory proteins; the early retarded (β) encoding proteins related to DNA replication, including thymidine kinase, and the late (γ) encoding structural proteins of the viral particle. The proteins that have been synthesized are classified into two categories: early proteins, which are synthesized immediately after infection and are required for viral nucleic acid replication; and the late proteins, synthesized later and include the viral coat glycoproteins (Murphy et al., 1999; Madigan et al., 2003; Walsh et al., 2013). In puppies, CaHV1 causes a brief and severe disease, which is characterized by viremia and 80% mortality in animals less than a week old. The surviving animals are left with irreversible cerebellar and articular sequelae. In the puppies two forms of presentation are observed: a super-acute form characterized by a fulminating mortality of asymptomatic type and another acute one (the most common) that initially presents with digestive disorders, anorexia, grayish-colored liquid stools, vomiting and continuous moaning, abdominal pain; then there are nervous symptoms of encephalomyelitis followed by coma and death (Murphy et al., 1999; Dumon and Mimouni, 2005). On the other hand, one of the facilitators of viral transmission is close contact between individuals, either through mucosal contact during intercourse, licking and caressing of mothers (Murphy et al., 1999; Dumon and Mimouni, 2005; Carter et al., 2006). Currently, canine herpesvirus is distributed worldwide, and its pathogenic potential is related to the age of the infected animals. This disease can occur in many shelters and canine hotels, stables and mainly in hatcheries, in which seroprevalence would exceed 90%. In these places, half of the seropositive animals would have reproductive disorders. There are cases of endemic herpesvirosis in hatcheries in which there have been cases of neonatal mortality; whereas in older puppies and adults there are different pathological signs, such as ocular and rhinopharyngeal conditions that manifests itself clinically as "kennel cough". In females, a slight vesicular vaginitis occurs, in addition, uterine infections can lead to miscarriage, poor birth and even infertility (Decaro et al., 2008; Dumon and Mimouni, 2005; Carter et al., 2006).

Diagnostic methods. This type of herpesvirosis could be detected clinically in a nursery when there are massive cases of perinatal mortality, followed by abortions, infertility and coughing of kennels (Anvik, 1991; Navarro et al., 2005). At necropsy, hemorrhagic petechiae are observed in the liver, spleen, lungs and kidneys, which correspond to characteristic lesions. While, from cuts of tissue affected by the virus, a rapid diagnosis could be made by immunofluorescence. However, the intranuclear inclusion bodies visible at the histological level are the pathognomonic lesions of this herpesvirus (Larenaset al., 1992; Navarro et al., 2005).

Polymerase Chain Reaction (PCR). Although, the viral isolation of CaHV1 in cell cultures is undoubtedly the standard test of excellence, unfortunately it takes at least a month. Therefore, its molecular detection has now been implemented through the PCR technique, a technique widely used around molecular biology (Mullis and Faloona, 1987).

This method to detect nucleic acids consists of three successive steps that together form a cycle: 1. *Denature*: the DNA is subjected to high temperatures (94-95°C) achieving the separation of the strands. 2. *Alignment*: the DNA is cooled (48-58°C), the synthetic oligonucleotides (primers) bind to sites near the region that will be amplified. 3. *Elongation*: carried out at intermediate temperature (72 ° C), the oligonucleotides of the mixture are incorporated into the extension product by the thermostable DNA polymerase. These three steps are repeated 30 or more times in order to obtain millions of copies of the DNA segment of interest, are repeated cycles that lead to an exponential synthesis of copies, which gives the technique a high sensitivity and specificity (Murphy et al., 1999), able to detect part of the genome of the pathogen of interest, from viral DNA present in affected organs and tissues (Erleset al., 2004; Ronsset et al., 2005; Dumon and Mimouni, 2005; Carter et al., 2006).

Multiplex PCR: Corresponds to a variant of the PCR that allows the simultaneous amplification of several sequences of interest in the same reaction, using more than one pair of primers. Therefore, this variant is more rigorous and demanding, maintaining the characteristics of sensitivity and specificity previously described (Chamberlain et al., 1988; Durzyńska et al., 2011).

Situation in Chile. The first finding of CaHV1 dates to 1992, when its presence was suggested based on the perinatal death of some puppies that showed the presence of petechiae in organs such as liver and kidney, as well as many intranuclear eosinophil inclusion bodies. The characteristic cytopathic effect of this virus was observed in the cultures of the equine kidney cell line occupied in that study (Larenaset al., 1992). Then a national isolate named RP5 (Navarro et al., 2005) was detected and biologically characterized. Since then, there are no other reports regarding any other viral characterization or any diagnostic tool in this regard. With this

background, in this report the simultaneous detection of two fragments of the gene that codes for a specific glycoprotein of CaHV1 was made: the glycoprotein B (gB). The gB gene consists of 2640 bp and the fragments used in this study are of 450 and 1286 bp. (Genbank, 2018)

This glycoprotein facilitates entry into the target cell in infections caused by free virions or infection due to cell-to-cell spread (Mettenleiter and Spear, 1994). This will contribute to the molecular confirmation of the presence of CaHV1 in Chile and, in addition, together with two other parallel Title Reports that contemplate different PCR variants (nested and conventional), will allow this procedure to be implemented as a quick and useful diagnostic technique for the control of this disease in Chile.

II. Material and Methods

Virus isolation: The virus used in this report was the RP5 national isolate (infected cell culture supernatant) maintained at -20°C in the laboratory of the Animal Virology Unit of the Faculty of Veterinary and Animal Sciences of the University of Chile. Negative controls included DNA from *Salmonella* Enteritidis and cDNA from the canine distemper virus, and nuclease-free water (NFW) was used as reagent control.

Detection of the glycoprotein B (gB) gene of CaHV1 by PCR. Primers to be used in multiplex PCR (PCR 3): two pairs of designed primers were used to separately amplify 2 fragments of the gB gene (PCR1 and PCR2), commissioned to the BIOSCAN company for its preparation: **PCR1:** GBN3 5'-TAATTCATATGTCCTTTT-3' and GBN4: 5'-GTCCTGTATCTTCTAACTCTGCT-3' (size 1286 bp; Erles et al., 2004); **PCR2:** RP1 5'-CCTAAACCTACTTCGGATGA-3' and RP2 5'-GGCTTTAAATGAACTTCTCTGG-3' (size 450bp, Ronsse et al., 2005)

Reaction mixture: A commercial kit (2X PCR Master Mix, Fermentas®), containing thermostable polymerase, deoxynucleotide triphosphates (dNTPs), reaction buffer and MgCl₂ was used. In a 0.2mL Eppendorf tube, 25µL of Master Mix, 5µL of each of the primers and 5µL of the DNA sample were added, obtaining a total volume of 50µL.

DNA amplification: For the determination of the optimum alignment temperature of the starters used, a thermal gradient thermocycler was used (Px2ThermalCycler, ThermoElectronCorporation), facilitated by Dr. Pedro Smith, of the Department of Animal Pathology of our Faculty, which allowed to perform the alignment stage at a known and individual temperature for each column of thermocycler wells. Thus, the PCR protocol used contemplated an initial denaturation at 94°C for 2 minutes and then 35 cycles that included denaturation at 94°C for 60 seconds, alignment for 60 seconds and extension at 72°C for 60 seconds. Finally, the final elongation at 72 °C for 10 minutes. The temperature range considered twelve degrees (40°C → 51°C) (*)

Stage	Temperature(°C)	Time(s)	Cycles
Initialdenaturation	94	120	1
Denaturation	94	60	35
Annealing	40-51(*)	60	
Elongation	72	90	
Final elongation	72	600	1

(Erles et al., 2004; Ronsse et al., 2005)

Visualization of amplified products. It was performed by electrophoresis in 2% agarose gel (Winkler ®) in Tris-borate buffer (90mM Tris-borate, 10mM EDTA) as solvent. The PCR product was mixed (6: 1) with a commercial loading product (6X MassRulerLoadingDyeSolution, Fermentas ®), which possesses glycerol and bromophenol blue to check the progress of migration of the DNA bands. Subsequently, an aliquot of 5 µL of this mixture was deposited in the respective well of the gel. Electrophoresis was carried out at 100V for 40 minutes. As a molecular size marker, a standard containing DNA fragments between 100 and 2000 bp (HyperLadder II, Bioline ®) was used. After electrophoresis, the gel was incubated in ethidium bromide (0.5 µg / mL) (Fermelo ®) and then placed in an ultraviolet transilluminator (TransilluminatorUVP ®) where it was photographed.

Analysis of results. The determination of the optimal temperatures and times of alignment and elongation of the multiplex PCR implemented, was carried out by visually comparing the photographic evidences (fluorescence of obtained amplicons); understanding as optimal, when presenting both fragments a similar fluorescence and band size. **Biosecurity measures.** The laboratory work was carried out according to the biosafety levels established for the Microbiology and Animal Virology laboratories, such as: use of clean material, correct elimination of waste and the use of closed apron and gloves in practical work. The visualization of the amplified product involved the use of ethidium bromide and a UV light transilluminator. Due to this, at the time of visualizing the gel an acrylic plate and glasses with UV filter were used. Subsequently, the elimination of the gel submerged in ethidium bromide contemplated its incineration, since the chemical compound mentioned has -among others- mutagenic properties.

III. Results

When analyzing the amplicons generated by the temperature gradient thermal cycler in the 2% agarose gel electrophoresis and its subsequent visualization under UV light, the presence of two DNA bands of around 450 and 1300 bp in the corresponding lanes was evidenced.

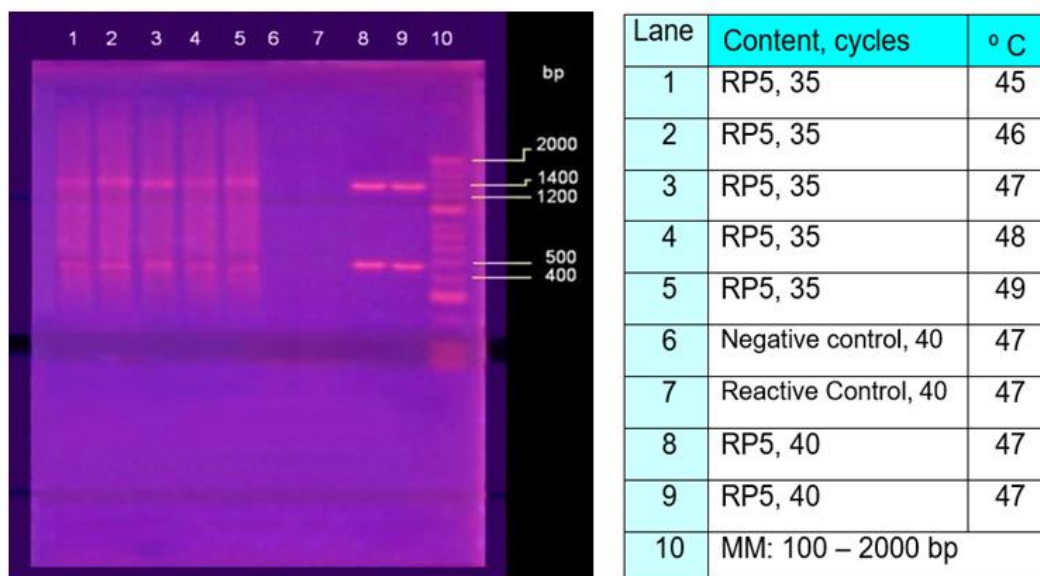


Figure 1

Gel electrophoresis of 2% Agarose. Multiplex PCR protocol. Canine herpes virus type 1:RP5 is native isolate.

However, the most intense bands were observed for the temperature of 47°C, with which this temperature could be defined as the optimum alignment temperature. From Figure 1, it is possible to point out that while preserving the initial conditions (annealing at 47° C, 60 s, elongation at 90 s), the increase in the number of cycles (from 35 to 40) make it possible to obtain even sharper fluorescent DNA bands. Figure 1 summarizes the results obtained in the implementation of this Multiplex PCR protocol.

IV. Discussion

For the diagnosis of CaHV-1 infection, both clinical examination in live animals, as well as necropsy and histopathological studies in dead animals are used. Although these methods suggest the presence of the virus, they cannot be definitive diagnoses. Therefore, the definitive method of choice is still viral isolation, which in turn takes approximately one month. For this reason, a PCR protocol is obviously more advantageous since it has greater specificity, since it detects a specific DNA fragment of a pathogen; greater sensitivity, because each cycle generates an exponential increase in the product to be detected and finally, it should be noted how quickly the results are obtained (Erles *et al.*, 2004; Ronsee *et al.*, 2005; Dumon and Mimouni, P.2005; Carter *et al.*, 2006; Mullis and Faloona, 1987). In consideration of the above, in FAVET several methods have been implemented for the diagnosis of CaHV-1 that involve the conventional PCR technique: by detecting the UL37 gene (Fuentes, 2010), the gB gene (Carrasco, 2010), the gC gene (Vargas, 2013) and in parallel another study is under development that detected the thymidine kinase (TK) gene through the use of a nested PCR (Jara, 2018, data not yet published). These methods, together with that presented in this Title Report, which included a multiple PCR protocol that involved the simultaneous detection of two DNA fragments of approximate sizes of 450 and 1280 bp, are intended to contribute to the search for a definitive method of diagnosis molecular structure of CaHV-1 that may be useful in Small Animal Medicine. Based on the results obtained, it was established that for this multiplex PCR, an alignment temperature of 47° C, an alignment and elongation time of 60 and 90 s, respectively, and the repetition of the process 40 times (cycles), allowed the Obtaining the amplicons with greater specificity and quantity. The availability of an early detection technique for CaHV-1 by PCR would allow diagnosing and solving an infectious disease that currently involves affective problems and economic losses. Similarly, the high mortality observed in puppies younger than four weeks could be reduced, and infected adults could be detected to avoid crossing these animals and remove them from the hatchery. On the other hand, knowing the sanitary status of a breeding place for dogs of high genetic value, would avoid large losses in the future, due to neonatal deaths and / or abortions.

V. Conclusion

Considering the results, it can be concluded that the multiple PCR protocol implemented was successful and that between the molecular versions of CaHV-1 diagnostic in FAVET, it could be the method of choice, considering its greater specificity, when simultaneously detecting two fragments of the glycoprotein B gene.

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