

**RT-PCR DETECTS CANINE DISTEMPER VIRUS IN ASYMPTOMATIC AND
NON-VACCINATED PUPPIES.**

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ABSTRACT

The objective of the present study was to use reverse transcription-polymerase chain reaction (RT-PCR) for canine distemper virus screening in puppies with asymptomatic canine distemper. Blood samples were taken from 12 non-vaccinated asymptomatic puppies, 10-45 days of age; of mixed breeds, ages, and sexes. Vero cells infected with canine distemper virus strain *Lederle* were used as the positive control. Using acid guanidinium thiocyanate-phenol-chloroform extraction, RNA was isolated and treated with a DNA-free™ kit (Ambion Inc., Foster, California, USA). Primers specific to the nucleocapsid protein coding region gene of canine distemper virus were designed and were able to amplify a fragment of 319 bp. Another target fragment of canine S26 (75 bp) was utilized as the endogenous control. Eight animals (67%) were positive and 4 (33%) were negative in a total of 12 animals analyzed. In conclusion, accurate diagnosis for canine distemper virus in early stages of infection using RT-PCR enhances identification of any infected puppies to be quarantined and prevents spread of disease.

INTRODUCTION

Canine distemper (CD) is one the most severe infectious diseases worldwide, can affect animals of all ages, but puppies are more susceptible at weaning, especially those less than three months of age, with high morbidity and mortality (Baker et al. 1959, Appel & Gillespie 1972, Appel 1987). The etiological agent is canine distemper virus (CDV) which belongs to the genus *Morbillivirus* within the family *Paramyxoviridae*. It is an RNA enveloped negative single strand virus that causes persistent infections in animals and cell cultures (Appel 1987). Many members of the *Canidae* family are susceptible to CDV (Greene & Appel 2006), and it is one of the most fatal illnesses in dogs (Kajita et al. 2006). It is comparatively rare in many developed countries, being well controlled through vaccination using the attenuated live virus (Greene & Schultz 2006).

Canine distemper is a systemic disease, acute or subacute, that causes respiratory, gastrointestinal and central nervous system infections (Appel & Summers 1995). The virus is present in all body excretions during the systemic phase. Clinical signs vary with the severity of the disease (Chappuis 1994), whereas some animals are asymptomatic (Cornwell et al. 1965). Diagnosis of CD in acute or subacute form had been done usually

based on clinical signs and history in unvaccinated puppies, but sometimes difficult as clinical signs may be confused with other canine respiratory or enteric diseases (Baumgärtner 1993, Moritz et al. 2000).

Accordingly, it is important the use of rapid, sensitive, and specific of CDV detection to start measures therapeutical and prophylactic. The objective of the present work was to use reverse transcription-polymerase chain reaction (RT-PCR) for CDV detection in peripheral blood samples of asymptomatic puppies not vaccinated for CD.

MATERIAL AND METHODS

Animals and sample collection.

Blood samples were collected from the jugular vein of 12 health puppies, non-vaccinated for CD, ranging in age from 10 to 45 days, mixed breeds and sexes. Blood samples (300 µL) were maintained in 1,000 µL of denaturation solution (Chomczynski & Sacchi 2006), in two-mL (Axigen scientific, INC., Union, CA, USA) microtubes. Samples were transported in dry ice and processed immediately in the laboratory. All procedures and protocols used in this work were approved by The Universidade Federal de Minas Gerais Animal Experimentation Committee.

Positive control.

VERO cells (*Chlorocebus aethiops* (African green monkey) kidney) infected with strain *Lederle* of canine distemper virus were used as the positive control. Vero cells were seeded on a 25 cm² flasks (12.5 x 10⁵ cells/mL) and incubated at 37°C in 5% CO₂ for 24 h in Modified Eagle's Medium (MEM) (Sigma, Chemical Co. St. Louis, MO, U.S.A) supplemented with 5% fetal bovine serum (FBS) (Sigma, Chemical Co. St. Louis, MO, U.S.A), penicillin (200UI/mL), streptomycin (200µg/mL), and anfotericina B (50µg/ml). For an additional 24 h, cells were kept in MEM to maintain cell cycle stage, after which, semi-confluent monolayer cultures were infected with CDV strain *Lederle* at a multiplicity of infection (MOI) of 0.1 and were maintained at the same conditions for additional 20 hours when cells were harvested for the RNA extraction experiments.

RNA isolation of and reverse transcription (RT).

RNA was isolated from 300 µL of either whole peripheral blood homogenate or CDV infected VERO cells using acid guanidinium thiocyanate-phenol-chloroform

extraction (Chomczynski & Sacchi, 2006). Samples were treated with a TURBO DNA-free™ kit (Ambion Inc., Foster, California, USA). Reverse transcription reaction was performed, as follows: 1 µg of RNA reversely transcribed in a 50 µL reaction mixture, containing 0.2 µL (40 U) of Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen Inc., Carlsbad, CA, USA), 2.5 µL (7.5 pmols) of each reverse oligonucleotide primer (Table 1), 4.5 µL of dNTP mix (250 µM each), 1.5 µL of 5X RT buffer, 1.2 µL of 20 mM DTT, and RNase-free water to complete the final volume. Reaction was incubated at 42°C for 1 hour, placed on ice and cooled to 4°C, and either used immediately for PCR or kept at -80°C for later used.

PCR for canine distemper virus.

Canine distemper virus nucleocapsid cDNA and housekeeping gene S26 were amplified by conventional PCR, using positive control cDNA samples (CDV infected VERO cells) and dog cDNA samples. Specific primers were designed for gel-based RT-PCR using the sequences obtained in GeneBank, through the Blast program (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>). Subsequently, all the sequences were analyzed using the Integrated DNA technologies website (<http://www.idtdna.com>) and specific primers that amplified a 319 bp amplicon for CDV and 75 bp for S26 were designed (Table 1). The conventional PCR reaction occurred under the following cycling conditions: denaturation at 95°C, annealing at 56°C and extension at 72°C for 40 cycles. During the PCR reaction, 1µL of cDNA was used in a final volume of 15 µL, with 4.9 µL of H₂O mili-Q, 3 µL of 5x buffer (Phoneutria Laboratory, BH, MG, Brazil), 5 µL of 25mM of MgCl₂ (Phoneutria Laboratory, BH, MG, Brazil), 1.5 µL dNTP-mix, 0.75 µL (10 pmols) of each primer and 0.125 µL (2.5 units) Taq-DNA polymerase (Phoneutria Laboratory, BH, MG, Brazil). PCR products were analyzed in 8% polyacrylamide gel stained with silver nitrate.

Table 1 – Sequence of oligonucleotides primers used in conventional RT-PCR assays.

| Primer | Sequences 5' - 3' | Amplicon size (bp) |
|-----------------|-----------------------------------|--------------------|
| S26 (forward) | 5' - CGTGCTTCCCAAGCTGTACGTGA - 3' | 75 pb |
| S26 (reverse) | 5' - CGATTCCGGACTACCTTGCTGTG - 3' | |
| CDV01 (forward) | 5' - CAGCACCGTACATGGTTATC - 3' | 319 pb |
| CDV02 (reverse) | 5' - GATTGCTTAGGACCAGTAGC - 3' | |

RESULTS

RT-PCR results confirmed the internal control S26 amplification of 75 bp in all samples, and also CDV amplification of 319 bp in positive animals. Eight (67%) puppies CDV positive, and four (33%) negative in a total of twelve animals analyzed (Fig. 1).

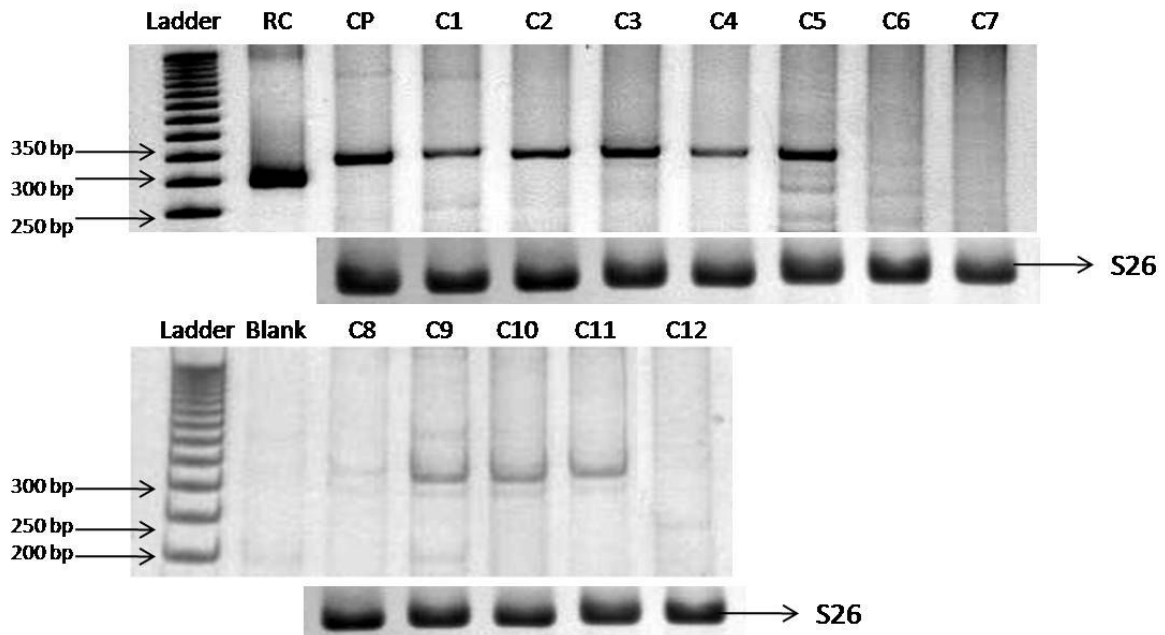


Figure 1 - 8% polyacrylamide eletrophoresis gel, stained with silver nitrate. (B) blank; Ladder - 50 bp ladder; (RC) PCR reaction control; (PC) positive control; samples – C1-C5, C9, C10 and C11 are CDV positive (67%), and C6, C7, C8 and C12 are CDV negative (33%).

DISCUSSION

Worldwide incidence of CDV infection is responsible for high mortality in puppies, mainly those less than 3 months of age, concurrent with maternal antibody loss after weaning (Baker et al. 1959, Appel et al. 1994, McCaw et al. 1998, Greene 1990). In Brazil, canine distemper is one of the most important infectious diseases that cause death in dogs. Epidemiological studies developed in Rio Grande do Sul (RS), Brazil by Headley & Graça (2000), showed that CD is an endemic disease responsible for up to 7% of clinical cases and 12% of deaths in dogs. In addition, Fighera et al. (2008) demonstrated that the prevalence of infectious diseases in post mortem dogs diagnosis are 35%, and CD is frequently diagnosed in adults (12.3%), and are the second disease more detected in puppies (17.2%), occurring not often in old dogs (2.8%). Bentubo et al. (2007) also demonstrated that infectious diseases are the main cause of death in dogs from São Paulo, Brazil. It is believed that the high prevalence of infectious and parasite diseases as a death cause in dogs are related with a non efficiency coverage of vaccination programs in Brazil most likely due to poor knowledge of the disease among dog owners (Fighera et al. 2008, Bentubo et al. 2007), the opposite it is observed in The United States and others countries in Europe where large amount of dogs has been vaccinated annually (Greene & Schultz 2006).

Clinical diagnosis is complex due to great variation in clinical manifestations and some techniques used for detection are neither sufficiently sensitive nor specific, generating conflicting results, most often in the subacute and chronic phases of the disease. However, some problems arise in differentiating CD from other diseases (Baumgärtner 1993, Moritz et al. 2000). Therefore, the use of a rapid, sensitive, and specific *anti-mortem* method of CDV detection is important.

Serological assessment has not been useful in diagnosis because high titres of anti-CDV antibodies may be the result of previous vaccination, yielding a false positive diagnosis (Baumgärtner 1993, Józwick & Frysmus 2005). Moreover, clinical or subclinical

infection can result in low antibody titres resulting in a false positive diagnosis (Baumgärtner 1993, Józwik & Frysmus 2005).

In our study, the RT-PCR assay results were useful tool for *anti-mortem* diagnosis of CD. Several attempts have been made to improve diagnosis of CD and several conventional RT-PCR assays have been developed that allow effective virus detection *in vivo* with high levels of sensitivity and specificity (Shin et al. 1995, Frisk et al. 1999). It is possible to detect viral nucleocapsid mRNA through RT-PCR (Del Puerto 2006), an important molecular tool in detection of acute and chronic infection, and *post-mortem* evaluation of CDV in different biological samples (Gordon et al. 1993, Frisk et al. 1999, Kim et al. 2001, Hoyland et al. 2003, Gebara et al. 2004).

Some infected dogs with CDV can be asymptomatic, acting as a reservoir of the virus (Cornewell et al. 1965), contributing to the endemic character and the increased incidence of CD. In our study the RT-PCR was a sensitive and effective method for indentifying CDV infected puppies permitting isolation of infected puppies from non-infected ones and preventing the spread of canine distemper virus.

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