

# Evaluation of RT-PCR and hemi-nested RT-PCR in brain samples from dogs with neurologic signs compatible with distemper

## *Avaliação das técnicas de RT-PCR e heminested RT-PCR em cérebros de cães com sinais neurológicos compatíveis com cinomose*

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### Abstract

The diagnostic value of RT-PCR and hemi-nested RT-PCR (hnRT-PCR) was compared in brain samples of dogs presenting neurological signs compatible with canine distemper. Samples of central nervous system (CNS) were collected from 68 dogs and tested by direct immunofluorescence test (RIFD) and, independent of the results, they were stored at -20°C for at least three years. They were submitted to the RT-PCR and hnRT-PCR techniques aiming to determine the gene responsible for the viral nucleoprotein decoding. Fifty-nine samples were positive for RIFD, 40 for RT-PCR ( $Kappa = 0.358$ ) and 54 for hnRT-PCR ( $Kappa = 0.740$ ). All nine RIFD negative samples were also negative for RT-PCR and hnRT-PCR. In spite of the storage duration and proper sample conditions, the estimated accordance between hnRT-PCR and RIFD demonstrated that hnRT-PCR technique can be applied in retrospective studies.

**Keywords:** Canine distemper. hnRT-PCR. Direct immunofluorescence. *Kappa index*.

### Resumo

Foi comparado o valor diagnóstico das técnicas de RT-PCR e heminested RT-PCR (hnRT-PCR) em amostras de cérebro de cães com sintomatologia nervosa compatível com cinomose. Fragmentos do sistema nervoso central (SNC) colhidos de 68 animais foram testados pela Imunofluorescência direta (IFD) e, independentemente do resultado, foram armazenados a -20°C por pelo menos três anos. Após esse período, foram submetidos a RT-PCR e a hnRT-PCR com oligonucleotídeos iniciadores direcionados ao gene codificador da nucleoproteína N. As proporções de resultados positivos/examinados foram: 59/68 para a IFD, 40/68 para a RT-PCR ( $Kappa = 0,358$ ) e 54/68 quando associada à heminested PCR ( $Kappa = 0,740$ ). Houve nove resultados negativos nas três técnicas empregadas. Os resultados do coeficiente Kappa entre a IFD e hnRT-PCR demonstram que apesar das condições de armazenamento, a hnRT-PCR pode ser utilizada em estudos retrospectivos.

**Palavras-chave:** Cinomose. RT-PCR. Imunofluorescência direta. Coeficiente *Kappa*.

Canine distemper is a multisystemic disease that occurs in domestic (GREENE; VALDEVELDE, 2012) and wild animals (JORGE et al., 2010). The disease is caused by a RNA virus, *Morbillivirus* genus, *Paramyxoviridae* family, with non-segmented, single stranded and negative sense genome (GREENE; VALDEVELDE, 2012).

The clinical signs of domestic dogs infected by canine distemper virus can vary from an unapparent infection to a severe neurologic disease. These variations are dependent on animal age, its immunological status and the viral strain (GREENE; VALDEVELDE, 2012).

The canine distemper virus (CDV) can be detected

using the direct immunofluorescence test (RIFD) (APPEL, 1969), which is based on the demonstration of viral inclusions in tissues or conjunctival smears (BRAZ, 2009), and by the polymerase chain reaction by reverse-transcriptase (RT-PCR), that detects

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Received: 28/11/2014

Approved: 14/10/2015

the viral genome, associated or not with a second amplification step, the hnRT-PCR (STANTON et al., 2002; JÓZWIK; FRYMUS, 2005). Both techniques can be used for *ante-mortem* (JÓZWIK; FRYMUS, 2005; AMARAL, 2007; BRAZ, 2009) and *post-mortem* diagnosis (KAPIL et al., 2008; ROSA et al., 2012).

The aim of this work was to evaluate the RT-PCR and the hnRT-PCR techniques in brain samples from dogs with neurologic signs compatible with distemper stored at  $-20^{\circ}\text{C}$  for at least three years.

Samples from the central nervous system (CNS) of 68 dogs were tested by RFID and, independent of their results, stored at  $-20^{\circ}\text{C}$  for at least three years and submitted to the RT-PCR and hnRT-PCR techniques using primers aimed at determining the gene responsible for the decoding of the viral nucleoprotein, N-gene (AMARAL, 2007).

The samples of CNS fragments were ground in sterile mortar and pestle and suspended as 10% (w/v) in DEPEC water. The extraction was performed using TRIzol<sup>®</sup> Reagent (Invitrogen) and the RNA was reverse-transcribed with the Moloney murine leukemia virus reverse transcriptase (M-MLV-RT; Invitrogen) using random primers according to the manufacturer's instruction.

The RT-PCR and hnRT-PCR enzymatic amplification was carried out in a final volume of 25  $\mu\text{L}$  containing 17.35  $\mu\text{L}$  of ultra-pure sterile water, 0.5  $\mu\text{L}$  of each dNTP's (10 mM), 2.5  $\mu\text{L}$  of 10X PCR reaction buffer, 1.25  $\mu\text{L}$  of each primer at 10 pmol/ $\mu\text{L}$ , 1.5  $\mu\text{L}$  of  $\text{MgCl}_2$  at 50mM; 0.15 U/ $\mu\text{L}$  of *Platinum*<sup>®</sup> Taq DNA Polymerase at 5U/ $\mu\text{L}$  (Life Technologies) and 2.5  $\mu\text{L}$  of cDNA. Amplification was performed at  $95^{\circ}\text{C}/5\text{min}$ , and 40 cycles at  $94^{\circ}\text{C}$  for 30s,  $56^{\circ}\text{C}$  for 30s,  $72^{\circ}\text{C}$  for 30s, and final extension at  $72^{\circ}\text{C}$  for 5min. The PCR products were analyzed on a 2% agarose gel after staining with ethidium bromide.

The proportions of positive results were 59/68 for RFID, 40/68 for RT-PCR ( $Kappa = 0.358$ ) and 54/68 for hnRT-PCR ( $Kappa = 0.740$ ). All RFID negative samples ( $n = 9$ ) were negative for RT-PCR and hnRT-PCR. The  $Kappa$  coefficient was calculated according to Sergeant (2013).

Several studies conducted using clinical samples from naturally infected animals presented better results when a second amplification was used (STANTON et al., 2002; JÓZWIK; FRYMUS, 2005; NEGRÃO; ALFIERI; ALFIERI, 2007; AMARAL, 2007; FRANCESCO et al., 2012). This fact can be due to the increase of analytical sensitivity when techniques are combined (STANTON et al., 2002; ARAÚJO et al., 2008; JÓZWIK; FRYMUS, 2005).

The false-negative results observed in the hnRT-PCR ( $n = 5$ ) can be explained by the activity of endogenous RNA released during sample storage at  $-20^{\circ}\text{C}$  (DE PAOLI, 2005) and/or by the heterogeneous distribution of the CDV in CNS of the naturally infected animals (SILVA, 2009; CARVALHO et al., 2012).

In spite of the storage duration and proper sample conditions, the comparative results for hnRT-PCR and RFID ( $Kappa = 0.740$ ) corroborate previous investigations conducted in similar conditions as the present study (STANTON et al., 2002; ARAÚJO et al., 2008), demonstrating that these techniques can be used in retrospective studies.

The presence of nine negative samples in the three methods confirms the specificity of the preconized molecular techniques and points to the need for regular and standardized differential diagnosis of several diseases that can promote neurological alterations in dogs.

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