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

Adriana CORTEZ[1]; Vanessa SOUZA[2], Telma FERNANDES[2]; Jane MEGID[2]

[1] Universidade de Santo Amaro, Medicina Veterinária, São Paulo – SP, Brazil
[2] Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia, Botucatu – SP, Brazil

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 (RFID) 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

Correspondence to:
Adriana Cortez
UNISA/Campus I – Curso de Medicina Veterinária
R. Prof. Enéas de Siqueira Neto, 340 - Jardim das Imbuias
CEP 04829-300, São Paulo, SP, Brazil
E-mail: acortez@unisa.br

Received: 28/11/2014
Approved: 14/10/2015

DOI: 10.11606/issn.1678-4456.v52i4p363-365

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°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°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® 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 of volume 25 µL containing 17.35 µL of ultra-pure sterile water, 0.5 µL of each dNTP's (10 mM), 2.5 µL of 10X PCR reaction buffer, 1.25 µL of each primer at 10 pmol/µL, 1.5 µL of MgCl2 at 50mM; 0.15 U/µL of *Platinum* ® Taq DNA Polymerase at 5U/ µL (Life Technologies) and 2.5 µL of cdna. Amplification was performed at 95°C/5min, and 40 cycles at 94°C for 30s, 56°C for 30s, 72°C for 30s, and final extension at 72°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 RIFD, 40/68 for RT-PCR (*Kappa* = 0.358) and 54/68 for hnRT-PCR (*Kappa* = 0.740). All RIFD 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°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 RIFD (*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.

References

AMARAL, H. A. **Detecção do vírus da cinomose pela técnica de RT-PCR em cães com sintomatologia neurológica**. 2007. 72 f. Tese (Doutorado em Clínica Veterinária) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2007.

APPEL, M. J. G. Pathogenesis of canine distemper. **American Journal of Veterinary Research**, v. 30, p. 1167-1174, 1969.

ARAÚJO, D. B.; LANGONI, H.; ALMEIDA, M. F.; MEGID, J. Heminested reverse-transcriptase polymerase chain reaction (hnRT-PCR) as a tool for rabies virus detection in stored and decomposed samples. **BMC Research Notes**, v. 1, n. 17, p. 1-6, 2008. Available from: <http://www.biomedcentral.com/1756-0500/1/17>. Viewed: 18 May 2013. doi: http://dx.doi.org/10.1186/1756-0500-1-17.

BRAZ, G. F. **Padronização e teste da técnica de imunofluorescência direta para o diagnóstico da cinomose canina**. 2009. 43 f. Dissertação (Mestrado) – Escola de Medicina Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, 2009.

CARVALHO, O. V.; BOTELHO, C. V.; FERREIRA, C. G. T.; SCHERER, P. O.; SOARES-MARTINS, J. A. P.; ALMEIDA, M. R.; SILVA JÚNIOR, A. Immunopathogenic and neurological mechanisms of canine distemper virus. **Advances in Virology**, v. 2012, Article ID 163860, 10 pages, 2012. Available from: <http://www.hindawi.com/journals/av/2012/163860/>. Viewed: 30 Apr. 2013. doi: http://dx.doi.org/10.1155/2012/163860.

DE PAOLI, P. Biobanking in microbiology: from sample collection to epidemiology, diagnosis and research. **FEMS Microbiology Reviews**, v. 29, n. 5, p. 897-910, 2005. Available from: <http://onlinelibrary.wiley.com/doi/10.1016/j.femsre.2005.01.005/pdf>. Viewed: 11 Sept. 2013. doi: http://dx.doi.org/10.1016/j.femsre.2005.01.005.

FRANCESCO, C. E.; FRANCESCO, D.; MARTINO, B.; SPERANZA, R.; SANTORI, D.; BOARI, A.; MARSILIO, F. Detection by hemi-nested reverse transcription polymerase chain reaction and genetic characterization of wild type strains of Canine distemper virus in suspected infected dogs. **Journal of Veterinary Diagnostic Investigation**, v. 24, n. 1, p. 107-115, 2012. Available from: <http://vdi.sagepub.com/content/24/1/107>. Viewed: 12 Sept. 2013. doi: http://dx.doi.org/10.1177/1040638711425700.

GREENE, C. E.; VALDEVELDE, M. Canine distemper. In: GRENE, C. E. (Ed.). **Infectious diseases of the dog and cat**. 4th ed. St. Louis: Elsevier Saunders, 2012. p. 25-42.

JORGE, R. S. P.; ROCHA, F. L.; MAY-JÚNIOR, A. J.; MORATO, R. G. Ocorrência de patógenos em carnívoros selvagens brasileiros e suas implicações para a conservação e saúde pública. **Oecologia Australis**, v. 14, n. 3, p. 686-710, 2010. Available from: <http://www.oecologiaaustralis.org/ojs/index.php/oa/article/view/oeco.2010.1403.06>. Viewed: 11 Sept. 2013. doi: http://dx.doi.org/10.4257/oeco.2010.1403.06.

JÓZWIK, A.; FRYMUS, T. Comparison of the immunofluorescence assay with RT-PCR and nested PCR in the diagnosis of canine distemper. **Veterinary Research Communication**, v. 29, n. 4, p. 347-359, 2005. Available from: <http://link.springer.com/article/10.1023%2FB%3AVERC.0000048528.76429.8b>. Viewed: 1 May 2013. doi: http://dx.doi.org/10.1023/B:VERC.0000048528.76429.8b.

KAPIL, S.; ALLINSON, R. W.; JOHNSTON III, L.; MURRAY, B. L.; HOLLAND, S.; MEINKOTH, J.; JOHNSON, B. Canine distemper virus strains circulating among North American dogs. **Clinical and Vaccine Imunnology**, v. 15, n. 4, p. 707-712, 2008. Available from: <http://cvi.asm.org/content/15/4/707.full>. Viewed: 15 June 2012. doi: http://dx.doi.org/10.1128/CVI.00005-08.

NEGRÃO, F. J.; ALFIEIRI, A. A.; ALFIERI, A. F. Avaliação da urina e de leucócitos como amostras biológicas para a detecção ante mortem do vírus da cinomose canina por RT-PCR em cães naturalmente infectados. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 59, n. 1, p. 253-257, 2007. Available from: <http://www.scielo.br/scielo.php?pid=S0102-09352007000100042&script=sci_arttext>. Viewed: 9 Aug. 2013. doi: http://dx.doi.org/10.1590/S0102-09352007000100042.

ROSA, G. N.; DOMINGUES, H. G.; SANTOS, M. M. A. B.; FELIPPE, P. A. N.; SPILKI, F. R.; ARNS, C. W. Detecção e análise filogenética do gene H de amostras do vírus da cinomose canina em circulação no município de Campinas, São Paulo. **Pesquisa Veterinária Brasileira**, v. 32, n. 1, p. 72-77, 2012. Available from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-736X2012000100012&lng=pt&nrm=iso&tlng=pt>. Viewed: 16 Feb. 2012. doi: http://dx.doi.org/10.1590/S0100-736X2012000100012.

SERGEANT, E. S. G. **Epitools epidemiological calculators**. Ausvet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease, 2013. Available from: <http://epitools.ausvet.com.au>. Viewed: 9 Sept. 2013.

SILVA, M. C. **Neuropatologia da cinomose canina**. 2009. 220 f. Tese (Doutorado em Medicina Veterinária) – Programa de Pós-Graduação em Medicina Veterinária, Universidade Federal de Santa Maria, Santa Maria, 2009.

STANTON, J. B.; POET, S.; FRASCA JR., S.; BIENZLE, D.; BROWN, C. C. Development of a semi-nested reverse transcription polymerase chain reaction assay for the retrospective diagnosis of canine distemper virus infection. **Journal of Veterinary Diagnostic Investigation**, v. 14, n. 1, p. 47-52, 2002. Available from: <http://vdi.sagepub.com/content/14/1/47.long>. Viewed: 10 Sept. 2013. doi: http://dx.doi.org/10.1177/104063870201400109.