

Canine distemper virus detection in asymptomatic and non vaccinated dogs¹

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ABSTRACT.- Del Puerto H.L., Vasconcelos A.C., Moro L., Alves F., Braz G.F. & Martins A.S. 2010. **Canine distemper virus detection in asymptomatic and non vaccinated dogs.** *Pesquisa Veterinária Brasileira* 30(2):139-144. Departamento de Patologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Campus Pampulha, Belo Horizonte, MG 31270-901, Brazil. E-mail: helendelpuerto@hotmail.com

A quantitative real time polymerase chain reaction (PCR) revealed canine distemper virus presence in peripheral blood samples from asymptomatic and non vaccinated dogs. Samples from eleven domestic dogs with no signs of canine distemper and not vaccinated at the month of collection were used. Canine distemper virus vaccine samples in VERO cells were used as positive controls. RNA was isolated with Trizol®, and treated with a TURBO DNA-free kit. Primers were designed for canine distemper virus nucleocapsid protein coding region fragment amplification (84 bp). Canine β -actin (93 bp) was utilized as the endogenous control for normalization. Quantitative results of real time PCR generated by ABI Prism 7000 SDS Software showed that 54.5% of dogs with asymptomatic canine distemper were positive for canine distemper virus. Dissociation curves confirmed the specificity of the real time PCR fragments. This technique could detect even a few copies of viral RNA and identify subclinically infected dogs providing accurate diagnosis of this disease at an early stage.

INDEX TERMS: Asymptomatic, canine distemper, diagnosis, dogs.

RESUMO.- [Detecção do vírus da cinomose canina em cães assintomáticos e não vacinados.] A reação em cadeia da polimerase (PCR) em tempo real revelou a presença do vírus da cinomose canina em amostra de sangue de cães assintomáticos e não vacinados. Amostra de onze cães domésticos sem nenhum sinal clínico de cinomose e que não foram vacinados no mês da coleta de sangue foram utilizados para análise. Amostra vacinal do vírus da cinomose canina em células VERO foi utilizada como controle positivo. O RNA total foi isolado utilizando-se Trizol®, e tratadas com o Kit TURBO DNA-free. Os

iniciadores foram desenhados para amplificar a região do nucleocapsídeo viral com 319pb e 84pb para a PCR convencional e PCR em tempo real, respectivamente. O fragmento alvo da β -actina canina com 93pb foi utilizado como controle endógeno e normalizador. Resultados quantitativos da PCR em tempo real gerados pelo programa ABI Prism 7000 SDS demonstraram que 54,5% dos cães assintomáticos foram positivos para o vírus da cinomose canina. As curvas de dissociação confirmaram a especificidade dos fragmentos da PCR em tempo real. A detecção precoce do RNA viral é importante para a identificação de cães subclínicamente infectados e limitar a difusão da doença.

TERMOS DE INDEXAÇÃO: Assintomáticos, cinomose canina, diagnóstico, cães.

INTRODUCTION

Canine Distemper (CD) is a pantropic worldwide infectious disease caused by canine distemper virus (CDV), a member of the genus *Morbillivirus* within the family Paramyxoviridae. CDV has an enveloped virion containing

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a nonsegmented negative-stranded RNA genome, which comprises approximately 16 Kilobases (Kb). Six genes encode for a single envelope-associated protein (M), two glycoproteins (the hemagglutinin H and the fusion protein F), two transcriptase-associated proteins (the phosphoprotein P and the large protein L), and the nucleocapsid protein (N) that encapsulates the viral RNA (Moss & Griffin 2006). The H gene protein is used for attachment to receptors on the cell in the first step of infection, and an adequate host immune response against the H protein may prevent CDV infection (von Messling et al. 2001). After attachment, the F protein promotes fusion of the viral envelope with the host cell membranes, and also promotes membrane fusion between the host cells, with formation of syncytia (Lamb et al. 2006). The M protein links ribonucleoproteins with envelope proteins during virion assembly, and the P protein regulates transcription, replication, and the efficiency with which the nucleoprotein assembles into nucleocapsids (Moss & Griffin 2006). The nucleocapsid gene is considered a conserved region among different CDV strains, while H gene is subjected to higher genetic and antigenic variation than other CDV genes, the amino acid sequence vary by approximately 10% among different CDV strains (Martella et al. 2008). The linear arrangement and position genome of the six genes on CDV are respectively: N, P, M, F, H, and L.

Canine distemper (CD) is one of the most important viral diseases in dogs in the world (Krakowka et al. 1980a), causing morbidity and mortality in non-vaccinated dogs and also in animals that were previously vaccinated (Krakowka et al. 1980b, Tipold et al. 1992, Summers et al. 1995). In Brazil at least two studies (Bentubo et al. 2007, Figuera et al. 2008) have shown that canine distemper is the most important cause of death or euthanasia in dogs. In one of these studies (Figuera et al. 2008), 12.4% of the dogs died or were submitted to euthanasia due to infection by canine distemper virus.

Variable clinical signs may be present, making the clinical diagnostic difficult (Appel & Summers 1995). Contact among infected (clinic or subclinical) dogs maintains the virus within the dog population (Greene & Apple 2006), mainly puppies (3-6 mo) who are susceptible to infection, due to the loss of maternal antibodies (Krakowka & Koestner 1976). However, canine distemper is also a common disease in adult Brazilian dogs (between 1 and 9 years old) and corresponds to the main cause of death in dogs within this average age (12.4%) (Figuera et al. 2008).

Vaccination against CD has been used widely for decades, but CDV infection is still an important disease (Elia et al. 2006). Any febrile condition of puppies with multisystemic symptoms should consider CDV infection.

Several laboratory tests are available to confirm clinical CDV infection; however most of the commonly used tests may not be sensitive, specific, and quantitative enough to detect subclinical infection. Immunofluorescence (IF) on conjunctival, nasal, and vaginal smears can detect CDV

antigens only within 3 weeks after infection, when the virus is still present in the epithelial cells (Appel 1987), but it has low sensitivity and can generate false negative diagnoses. Virus isolation on cell lines from clinical samples is fastidious. ELISA assay may detect high antibody titers to CDV for several months after vaccination or after subclinical or clinical infection, turning false positive results (Martella et al. 2008). Molecular assays, such as RT-PCR (Frisk et al. 1999, Kim et al. 2001, Rzezutka & Mizak 2002, Gebara et al. 2004) are sensitive and specific, but not quantitative.

Canine subclinical infection by CDV contributes to the spread of the disease, requesting the development of a more sensitive diagnostic technique for early detection and quantification in asymptomatic animals and in those with subclinical infection. The quantitative real time PCR technique has been used for CDV detection and quantification in blood samples, conjunctive swabs, urine, and tissues in dogs showing clinical signs of disease, and also in infected VERO cells (Elia et al. 2006, Scagliarini et al. 2007). The objective of this study was to use quantitative real time PCR for CDV screening in peripheral blood samples of dogs with asymptomatic CD.

MATERIALS AND METHODS

Animals

Blood samples from fourteen dogs of different breeds, ages, and sexes were collected with the owners' permission, in Belo Horizonte, Minas Gerais, Brazil. Subclinical infection group had eleven animals with no clinical signs of any disease, and no vaccination during the last thirty days. To contrast that, blood samples from three other dogs with clinical signs of CD (including listlessness, decreased appetite, fever, and oculonasal discharge) were collected.

Positive control

Vaccine samples (Onderstepoort and Rockborn) cultivated in VERO cells were used as positive controls. They also allowed the construction of a dilution curve for the absolute quantification of the samples tested in real time PCR. Vaccine samples were kindly supplied⁵.

RNA isolation and reverse transcription (RT)

RNA was isolated from 300µl of either total peripheral blood homogenate or VERO cell CDV positive controls using Trizol^{®6}. Samples were treated with TURBO DNA-free kit⁷. Reverse transcription (RT) was performed. Briefly, 2µg of RNA were reversely transcribed in a 40 µl reaction mixture containing 40 U of Moloney Murine Leukemia Virus Reverse Transcriptase⁸, 25 pmols of each reverse oligonucleotide primer (Table 1), 4µl of dNTP mix (250µM each), 8µl of 5X RT buffer, 2µl of 20mM DTT, and RNAse-free water to complete the final volume. The

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⁶ Trizol[®], Invitrogen Corporation, Rua Breno Ferraz do Amaral 390/408, Jd. da Saúde, São Paulo, SP.

⁷ Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA, USA.

⁸ Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA, USA.

Table 1. PCR primers VCC01/VCC02 used in gel-based PCR and VCCIN/VCC01 used in real time used for CDV

Primers	Nucleotide sequence (nt)	Fragment size	mRNA position
FORBETADOG (forward)	5'-CACCTTCTACAACGAGCTGCG -3'	93 bp	(264-284)
REVBETADOG (reverse)	5'-ATCTTCTCACGGTTGGCCTTG-3'		(502-482)
VCCIN (reverse)	5'-TAGCATAACTCCAGAGCAATG -3'	84 bp	(1116-1096)*
VCC01 (forward)	5'-CAGCACCGTACATGGTTATC -3'		(1033-1052)*
VCC02 (reverse)	5'-GATTGCTTAGGACCAGTAGC -3'	319 bp	(1351-1332)*
AGT 1(RC) (forward)	5'-TCCACAGATCCGTGATGACTC-3'	298 bp	
AGT 2(RC) (reverse)	5'-GCAGCTCGCTGCCGATCCTC-3'		

*Sidhu et al. (1993).

reaction mixture was incubated at 42°C for 1h, placed on ice and cooled to 4°C, and either used immediately for PCR or kept at -80°C for later use.

Gel-based RT-PCR

Canine distemper virus nucleocapsid cDNA and canine housekeeping gene β -actin were amplified by conventional PCR, using positive control cDNA samples (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 93 bp for β -actin were designed (Table 1). Exon 2 of rat angiotensinogen (AGT), which amplified a 298 bp amplicon was used as PCR reaction control (RC). 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. Target amplicons of positive controls were agarose gel purified using Zymoclean™ Gel DNA Recovery Kit⁹, and quantified to create serial dilutions ranging from 1ng to 10⁻⁹ng to obtain a standard curve for the real time PCR absolute quantification. Primers pairs for CDV nucleocapsid gene were selected based on their conservation among different CDV strains.

Quantitative real time PCR

Absolute quantitative real time PCR was performed to detect fragments of 84 bp for CDV, within nucleocapsid gene; and 93 bp for canine β -actin. Specific primers were designed as described in gel-based RT-PCR (Table 1). Real time PCR was performed with an ABI Prism® 7000 Sequence Detection System¹⁰, using SYBR® Green Master Mix¹¹. PCR reactions were set up in an isolated room, and gloves, face masks, and barrier tips were used during all experiments. The final PCR mixture contained 1.5µl each of forward and reverse primers (final concentration 1.5pmols each), 2µl of cDNA from RT, 10µl of Sybr®Green Mix¹¹ and 5µl of PCR water, in a final volume of 20µl. Universal cycling conditions were as specified by ABI (2 min at 50°C, 10min at 95°C, 40 cycles of 15 s at 95°C, and 1min at 60°C). Cycle threshold values were determined by automated threshold analysis of ABI Prism software (v.1.0).

The amplification efficiency ($E=10^{-1/\text{slope}}$) was determined by the slope of the specific standard curve. The linearity was determined by regression coefficient (r^2 value ≥ 0.987) (Pfaffl 2001). The absolute quantitative expression level values were given in nanograms (ng) for each sample, calculated from standard curve dilution by the ABI Prism 7000 SDS Software. Dissociation curves were always analyzed to confirm gene target specificity.

RESULTS

Gel-based RT-PCR

The conventional PCR results confirmed CDV detection in positive controls (CDV vaccine samples - Onderstepoort and Rockborn - in VERO cells), in the three sick dogs (100%), and in three out of eleven asymptomatic dogs (27%), amplifying a fragment of 319 bp, and also the internal control β -actin of 93 bp (Fig.1). Agarose gel purification results in 8% polyacrylamide gel demonstrated the specificity of the CDV and β -actin fragments (Fig.2).

Quantitative real time PCR

The quantitative real time PCR was obtained by construction of standard curves for CDV and β -actin using purified standard amplicons of known quantities, which were used in serial dilutions of 1ng to 10⁻⁹ng. Standard curves demonstrated high linearity ($r^2 > 0.99$) for both targets studied, and the slopes were less than -2.0; that is, the efficiency ($E=10^{-1/\text{slope}}$) of all reactions was 100%. The quantification of CDV in each sample was given as ng of CDV/ng of β -actin. Dissociation curves were unique for each amplicon and confirmed gene target specificities (Fig.3). The quantitative real time PCR was able to detect

⁹ Zymoclean™, Zymo Research, 625 West Katella Avenue Suite 30, Orange, CA, USA.

¹⁰ ABI Prism® 7000 Sequence Detection System, Applied Biosystems do Brasil, Avenida do Café 277, Vila Guarani, São Paulo, SP.

¹¹ SYBR®Green Master Mix, Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA, USA.

Fig.1. Typical CDV gel-based PCR diagnostic test of total blood using VCC01/VCC02 primers. Samples are from positive controls CDV vaccine samples - Onderstepoort and Rockborn - in VERO cells (PC1 and PC2, respectively); 3 clinically suspected dogs (SD1-SD3); and 11 asymptomatic dogs (AD1-AD11). 3-5µl of the amplification reaction were applied and visualized by silver stained 8% polyacrylamide gel, after electrophoresis at 100V/50 min. CDV specific amplicon of 319 bp is indicated, and the β-actin 93 bp normalizer is shown for each sample. Ladder: 50 bp DNA ladder (0.3µg); RC: PCR reaction control (AGT) amplicon of 298 bp; Blank: no template negative control.

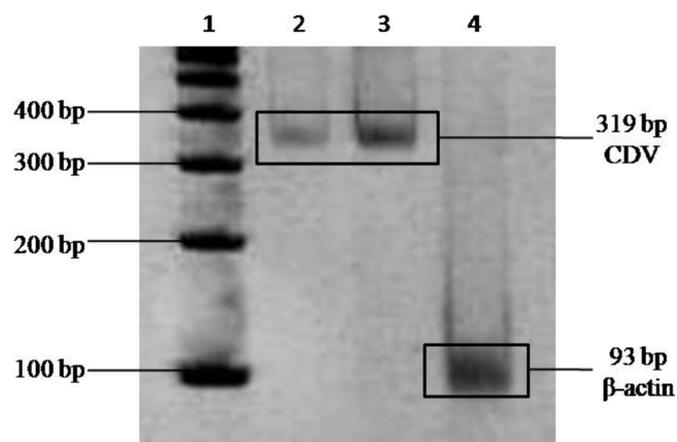
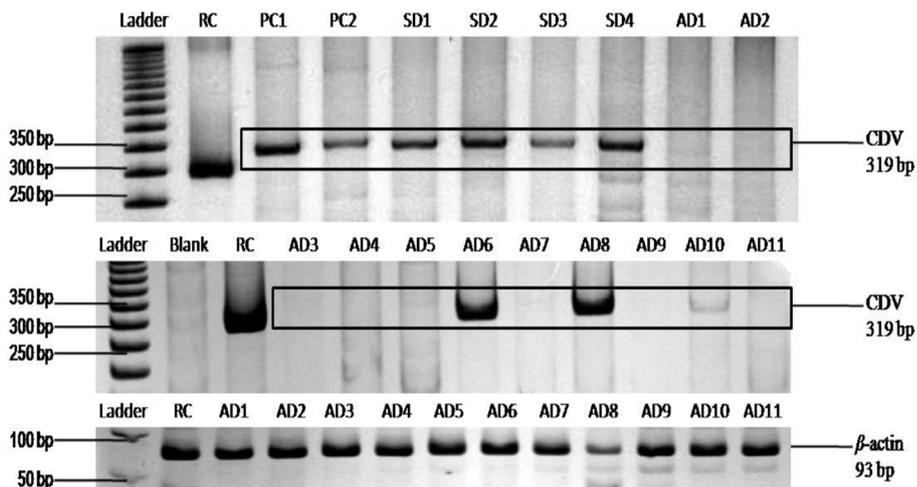


Fig.2. 8% polyacrylamide gel showing the purity and integrity of CDV and β-actin fragments, with 319 bp and 93 bp, respectively. 1: 2µl of 100 bp DNA ladder; 2: 15µl of CDV amplicon (Onderstepoort); 3: 15µl CDV amplicon (Rockborn) and 4: 15µl of β-actin fragment.

CDV in 6 out of 11 animals (54.5%) (Fig.4). Amplification of the internal control β-actin was observed in all samples and was used to normalize the CDV quantification in each sample.

DISCUSSION

Canine distemper (CD), one of the most fatal diseases in puppies, remains an important viral disease in the veterinary field. Unfortunately, there is still no effective therapy for CD, only symptomatic treatments (Kajita et al. 2006). CDV enters by the nasal or oral route and starts replication in the lymphoid tissues (Appel 1969), resulting in immunosuppression. The incubation period may range from 1 to 4 weeks or more. Transient fever reaches a peak 3 to 6 days after infection and is associated with the initial virus spread in the body. By days 6 to 9 after infection, CDV spreads by cell-associated viremia to the epithelial cells in most organs (Appel et al. 1982). If the dog develops

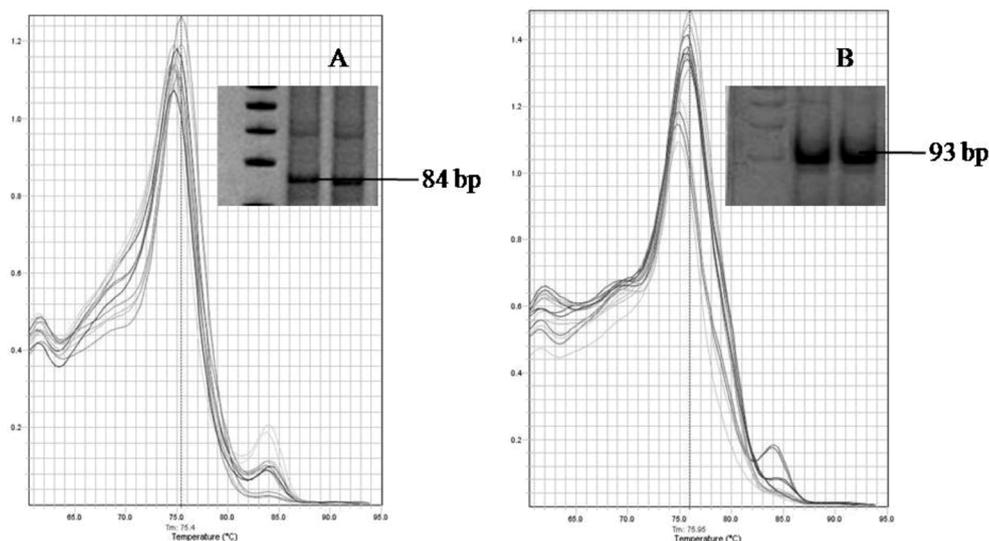


Fig.3. (A) and (B) shows the dissociation curve for CDV and β-actin, respectively, to check the specificity and size of the amplified fragments by real time PCR. 8% polyacrylamide gel confirms the CDV 84 bp fragment and β-actin 93 bp, after the real time PCR reaction.

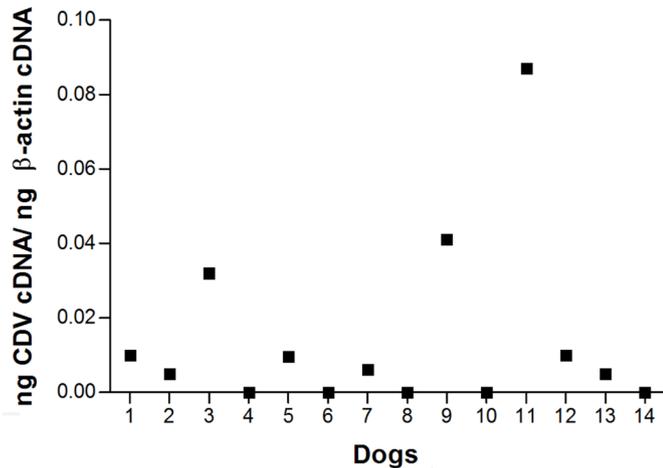


Fig.4. The CDV detection occurred in all 3 symptomatic dogs' samples tested (100%). Symptomatic dogs 1 (SD1), 2 (SD2) and 3 (SD3), with a viral amount of 0.041, 0.087 and 0.032ng, respectively. The CDV detection occurred in 6 out of 11 asymptomatic dogs' samples tested (54.5%). Asymptomatic dogs: 5 (AD2), 7 (AD4), 9 (AD6), 11 (AD8), 12 (AD9) and 13 (AD10), with a viral amount of 0.096, 0.0621, 0.041, 0.087, 0.01 and 0.003ng, respectively. Asymptomatic dogs: 4 (AD1), 6 (AD3), 8 (AD5), 10 (AD7) and 14 (AD11) with no CDV detection.

a strong immune response, the virus gets cleared from the tissues and the animal recovers from the infection. Dogs that fail to mount an immune response will show continuous virus replication and spreading massively throughout the body (Martella et al. 2008). Thus, infection relies on diseased and also on subclinically infected dogs.

A quantitative real time PCR assay using blood samples to detect asymptomatic infection in dogs would help to detect the virus during viremia, before it reaches epithelial cells and be detectable in animal secretions and excretions. So far, there is no data reporting the percentage of CDV infected dogs with subclinical CD on a population. Thus, it is urgent and necessary a sensitive, specific and quantitative assay to perform such epidemiological study.

The application of molecular techniques (for example, gel-based RT-PCR) in CDV diagnosis has been successfully used in different types of samples collected from animals clinically suspected of infection (Frisk et al. 1999, Kim et al. 2001, Rzeszutka & Mizak 2002, Gebara et al. 2004). It is a specific, practical and rapid tool in CDV diagnosis *ante mortem* considering the non-specific clinical signs of the disease, but it is not a quantitative and very sensitive assay.

Immunohistochemistry technique in naturally CDV-infected dogs has been used as an auxiliary tool in *post mortem* CDV detection (Sonne et al. 2009). The conventional RT-PCR combined with nested PCR demonstrated increased sensitivity of CDV detection (Kim et al. 2001), enabling the detection of CDV nucleoprotein (NP) in animals recently vaccinated with the attenuated virus and resulting in CDV detection in 4 out of 5 animals within 2 to 7 days of vaccination. However, no CDV

detection was observed after 14 days of vaccination (Kim et al. 2001). It is estimated that 25% to 75% of dogs susceptible to CD are subclinically infected and are transmitting the virus without any clinical sign of disease (Greene & Apple 2006).

In addition, asymptomatic dogs are not diagnosed and remain as a CDV reservoir. Hence, the use of gel-based PCR is useful but not enough to detect CDV in asymptomatic animals, because it is a qualitative and not a quantitative technique. Nowadays, studies with other viral diseases, such as HIV, have been done using the real time PCR technique to evaluate the efficiency of antiviral drugs, allowing for virus quantification during treatment (Malnati et al. 2008). Quantitative real time PCR based on the CDV nucleocapsid gene showed itself to be highly sensitive, capable of detecting 10^{-4} ng of virus cDNA and proving to be more sensitive than gel-based conventional PCR.

In addition, gel-based RT-PCR was able to detect CDV infection in 3 out of 11 (27%) asymptomatic dogs, while real time PCR assay was able to detect CDV infection in 6 out of 11 (54.5%) asymptomatic dogs, being more sensitive and quantitative than conventional RT-PCR assay. This confirms real time PCR as an important tool for virus quantification suitable for research and diagnostic purposes. Unfortunately, no information on later clinical history in those 6 animals, CDV positive in real time diagnosis, could be obtained. Further work is needed to evaluate canine distemper virus prevalence in asymptomatic dog's populations. This would include a larger sample and different seasonal sampling. A quantitative real time PCR reaction in asymptomatic dog's secretions would be helpful to answer this question. The detection of even a few copies of viral RNA is particularly useful for the identification of subclinically infected dogs that contribute to the diffusion of the disease; accurate diagnosis of this disease at an early stage is required to quarantine any infected animals and to prevent the spread of the disease.

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