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Detection of canine distemper virus in dogs by real-time RT-PCR

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Abstract

Canine distemper virus is the etiological agent of a severe disease in dogs and many other carnivores. Clinical diagnosis of canine distemper is difficult due to the broad spectrum of signs that may be confounded with other respiratory and enteric diseases of dogs. Accordingly, a laboratory confirmation is required for suspected cases. In this study a real-time RT-PCR assay was developed for detection and quantitation of canine distemper virus. The assay exhibited high specificity as all the negative controls (no-template-controls and samples from healthy sero-negative dogs) and other canine pathogens were not misdetected. Up to 1×10^2 copies of RNA were detected by the TaqMan assay, thus revealing a high sensitivity. Quantitative TaqMan was validated on clinical samples, including various tissues and organs collected from dogs naturally infected by canine distemper virus. Urines, tonsil, conjunctival swabs and whole blood were found to contain high virus loads and therefore proved to be suitable targets for detection of canine distemper virus RNA.

Keywords: Dog; Canine distemper virus; Fluorogenic PCR; Viral load

1. Introduction

Canine distemper virus is the etiological agent of a serious, often fatal, disease in dogs and many other carnivores (Appel, 1987). Canine distemper virus belongs to the genus *Morbillivirus*, family Paramixoviridae, that includes measles virus, phocine distemper virus and rinderpest virus. Morbilliviruses have enveloped virions and a negative-sense, single stranded RNA genome. The RNA encodes six structural proteins: two membrane glycoproteins, the fusion (F) and the hemagglutinin (H), the envelope-associated matrix (M) protein, the phosphoprotein (P), the large polymerase (L) and the nucleocapsid (N) protein.

Although vaccination against canine distemper has been used widely for many decades, this infection still represents an important disease for dogs. Targets of infection by canine distemper virus are mainly mucous membranes and lymphoid tissues. Following aerosol infection, the virus primarily replicates in lymphatic tissues of the respiratory tract and subsequently reaches various organs, including the cells of the lower respiratory and gastrointestinal tracts, the lymphoid organs, the urinary bladder and the central nervous system (Appel, 1987). This can result in either subclinical infection or a combination of respiratory, ocular, gastrointestinal, neurologic and cutaneous signs or lesions, that appear simultaneously or sequentially (Greene and Appel, 1998). Nervous signs may be present in the chronic form of distemper along with other manifestations, or may occur without any other signs (Appel, 1987). The broad spectrum of clinical signs, not dissimilar from the signs observed in other respiratory and enteric diseases of dogs, hampers clinical diagnosis of canine distemper (Jones et al., 1997) and renders necessary laboratory confirmation. Routine diagnosis of canine distemper virus by immunofluorescence (IF) is applied to various specimens, including conjunctival, nasal and vaginal smears, using polyclonal or monoclonal antibodies. This test is not sensitive and can detect canine distemper virus antigens only within 3 weeks after infection, when the virus is still present in the epithelial cells (Appel, 1987).

Serological methods, such as enzyme-linked immunosorbent (ELISA) and seroneutralization (SN) assays, have little diagnostic value because high titers of antibodies to canine distemper virus may be the result of previous vaccination or subclini-

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cal/clinical infection (Shin et al., 1995; Frisk et al., 1999; Kim et al., 2001).

A definitive diagnosis of canine distemper by virus isolation on canine cells is fastidious and time-consuming, taking several days to weeks, notably when applied to clinical specimens (Shin et al., 1995; Frisk et al., 1999; Kim et al., 2001). Recently, VERO cells expressing the canine signaling lymphocyte activation molecule were engineered that proved to be highly sensitive for virus isolation, showing cytopathic effect as early as 24 h after sample inoculation (Seki et al., 2003).

The contagious nature and the high mortality rates of canine distemper make it necessary to speed up the diagnostic procedure in order to quarantine infected dogs and start appropriate treatments early. Accordingly, a sensitive, specific and rapid method is desirable to detect even small amounts of virus early in infection. Reverse-transcriptase-polymerase chain reaction (RT-PCR) has been applied successfully to diagnosis of canine distemper virus (Frisk et al., 1999; von Messling et al., 1999; Rzeżutka and Mizak, 2002; Saito et al., 2006; Shin et al., 2004). The aim of this study was to develop a rapid, sensitive and specific real-time RT-PCR method to detect and quantify canine distemper virus in clinical specimens of naturally infected dogs. Real-time RT-PCR assays for canine distemper virus offer substantial improvements in virus detection and thus may render easier the control of canine distemper disease.

2. Materials and methods

2.1. Animals and sample collection

Canine distemper virus-positive samples (blood, conjunctival swabs and urines) from eight dogs (nos. 1–8) were collected during 2005. The dogs showed typical signs of canine distemper, such as fever, purulent ocular and nasal discharge, tonsillitis, bronchitis, gastroenteritis or neurological disturbance and diagnosis was confirmed by RT-PCR (Frisk et al., 1999). Archival tissue samples (brain and/or spleen) from seven additional dogs positive to canine distemper virus (nos. 9–15) were included in the study. The whole carcase from a canine distemper virus-positive dog (no. 16) was used for detailed investigation of canine distemper virus distribution in the various tissues and organs.

The negative controls used in the study included: (i) blood samples taken from two healthy dogs one day prior to vaccination; (ii) no-template-controls; (iii) samples containing other canine pathogens, i.e. CCoV type I and CCoV type II (Decaro et al., 2005a), reoviruses (Decaro et al., 2005b) and rotaviruses (Martella et al., 2001).

2.2. Preparation of RNA samples

Each sample was prepared for RNA extraction using commercial kits, depending on the examined probe. QIAamp Blood Mini Kit was used to isolate RNA from 1 ml of blood samples. QIAamp Viral RNA protocols (QIAamp Viral RNA, Qiagen S.p.A., Italy) were used to isolate total RNA from swabs (140 μl from 1 ml of transport medium used to resuspend the swabs in by vortexing) and from 140 μl of urine. RNA was extracted from tissues (25 mg) with the RNeasy Total RNA Kit (Qiagen S.p.A.). To rule out carryover contamination, negative controls included the RNA extracted from biological samples of healthy dogs as well as the RNA of other canine pathogens. RNA isolation was performed according to the manufacturer's instruction.

2.3. Design of primers and probe

Sequenced retrieved from the GenBank database were used to design primers and probe. Canine distemper virus conserved regions were identified by generating multiple sequence alignments using the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The H and F genes of canine distemper virus display a high rate of nucleotide variation that allows distinction of a number of genotypes or genetic clusters, according to a geographic pattern (Martella et al., in press). Accordingly, selection of primers was restricted to the N protein-encoding gene, that is subjected to a lower degree of variation (Rima et al., 1995). Primers and TaqMan probe were designed using Beacon Design software version 2.0 (Premier Biosoft International, Palo Alto, CA, USA) to amplify a conserved 83-bp fragment within the N protein region. Primers and probe were synthesized by MWG Biotech AG (Ebersberg, Germany). The TaqMan probe was labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end. The position and sequence of the primers and probe used for TaqMan RT-PCR amplification are reported in Table 1.

2.4. Generation of standard RNA

An 880 nt-long RT-PCR product encompassing the targeted region was prepared using canine distemper virus strain Onderstepoort. The sequence of the primers are the follows: P for 5′-ATGAAACGATCCCCAGGG-3′; P rev 5′-ACTGATGTAACACTGGTCT-3′. The resulting RT-PCR product was cloned into pCR4-TOPO vector (TOPO TA cloning, Invitrogen, Milan, Italy) and in vitro transcribed with RiboMAXTM Large Scale RNA Production System-T7 (Promega Italia, Milan, Italy) from the T7 promoter, according to the manufacturer's guidelines. Residual DNA was eliminated by DNAse treatment and the transcript was purified with Rneasy columns (Qiagen S.p.A., Italy) and quantified by spectrophotometrical analysis. Ten-fold dilutions of the RNA transcripts were made in TE (Tris–HCl, EDTA, pH 8.0) buffer containing 30 μg carrier RNA (tRNA from *Escherichia coli*, Sigma–Aldrich Srl, Milan, Italy) per ml. Aliquots of each dilution were frozen at −70 °C and used only once.

2.5. Reverse transcription

Triplicate of the standard dilutions and RNA templates were subjected simultaneously to reverse transcription (RT). One microliter of each triplicate of standard dilutions or template RNA was reverse transcribed in a reaction volume of 20 μl

Table 1
Oligonucleotides used in fluorogenic and conventional RT-PCR assays for canine distemper virus

Primer/probe	Sequences 5′–3′	Sense	Position	Amplicon size (bp)
p1[a]	ACAGGATTGCTGAGGACCTAT	+	769–789	287
p2[a]	CAAGATAACCATGTACGGTGC	–	1055–1035	
CDV-F[b]	AGCTAGTTTCATCTTAACTATCAAATT	+	905–931	87
CDV-R[b]	TTAACTCTCCAGAAAACTCATGC	–	966–987	
CDV-Pb[b]	FAM-ACCCAAGAGCCGGATACATAGTTTCAATGC-TAMRA	–	934–963	

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine. Oligonucleotide positions are referred to the sequence of canine distemper virus strain Ondesterpoort.
[a] Coventional RT-PCR (Frisk et al., 1999).
[b] Fluorogenic RT-PCR.

containing PCR buffer 1× (KCl 50 mM, Tris–HCl 10 mM, pH 8.3), $MgCl_2$ 5 mM, 1mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), Rnase Inhibitor 1 U, MuLV reverse transcriptase 2.5 U, random hexamers 2.5 U. Synthesis of c-DNA was carried out at 42 °C for 30 min, followed by a denaturation step at 99 °C for 5 min.

2.6. Real-time assay for canine distemper virus

For the real-time assay, 20 μl of c-DNA was added to 30 μl of reaction master mix. The master mix consisted of 25 μl of IQTM Supermix (Bio-Rad Laboratories Srl, Milan, Italy), 600 nM of each primer (P for and P rev), 400 nM of probe CDV-Pb. Fluorogenic PCR was carried out in a LightCycler instrument (i-Cycler iQTM Real-Time Detection, Bio-Rad Laboratories Srl) with the following steps: activation of iTaq DNA polymerase at 95 °C for 10 min and 45 cycles consisting of denaturation at 95 °C for 15 s, primer annealing at 48 °C for 1 min and extension at 60 °C for 1 min.

Standard RNA (5000 copies/ml of faecal suspension) used in a real-time RT-PCR assay for avian influenza virus (Di Trani et al., in press) was employed as an internal control (IC) to confirm the successful extraction of RNA, conversion to c-DNA and the TaqMan PCR reaction. The fixed amount of IC added to each sample had been calculated to give a mean threshold cycle (C_T) value in the real-time RT-PCR assay of 35.33 with a S.D. of 0.79, as calculated by 30 separate runs. Real-time PCR for IC detection was carried out in a separate run, using primers M-Flu1 (CTTCTAACCGAGGTCGAAACGTA) and M-Flu2 (GGATTGGTCTTGTCTTTAGCCA) and minor groove binder probe M-Fluprob (FAM-CTCGGCTTTGAGGGGGCCTGA-MGB). Samples in which the C_T value for the IC was > 36.91 (average + 2S.D.) were excluded from analysis.

2.7. Gel-based RT-PCR for canine distemper virus

The PCR assay amplifies a 287-bp region from the N gene (Frisk et al., 1999). The position and sequence of the primers are reported in Table 1. Samples were extracted as described above. Briefly, for amplification the GeneAmp RNA PCR kit (Applied Biosystem, Applera, Italy) was used. RT was performed at 42 °C for 30 min. After inactivation of MuLV reverse transcriptase, the PCR mix (1 μM of each primer) was added, followed by denaturation at 94 °C for 10 min and 45 cycles consisting of denaturation at 94 °C, annealing at 59.5 °C for 2 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The RT-PCR products were analyzed on 2% agarose gel after staining with ethidium bromide.

3. Results

3.1. Dynamic range, specificity and reproducibility of the real-time RT-PCR assay

Serial 10-fold dilutions ranging from 1×10^0 to 1×10^8 copies of control RNA were reversed transcribed. cDNAs were then used to determine the detectability and the linearity of the assay (Fig. 1). C_T values were measured in triplicate and were plot against the known copy number of the standard samples. The generated standard curve covered a linear range of seven orders of magnitude and showed linearity over the entire quantitation range (slope = −3.831), providing an accurate measurement over a very large variety of starting target amounts. The coefficient of linear regression (R_2) was equal to 0.997 and the PCR efficiency ranged around 82.4%.

Experiments were undertaken to asses diagnostic criteria such as specificity and reproducibility. Samples known to be positive for other infectious agents were run in this assay to

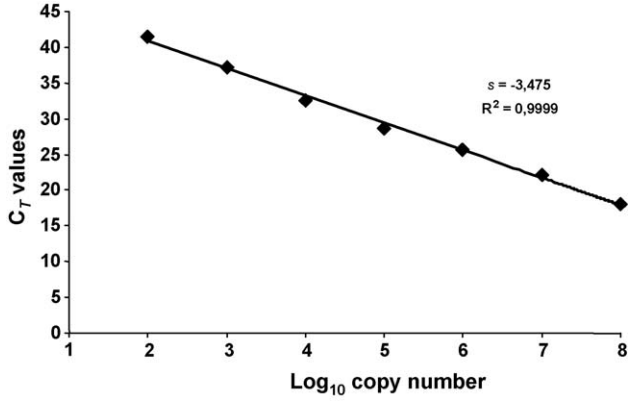

Fig. 1. Standard curve of the real-time RT-PCR assay for canine distemper viirus. Ten-fold dilutions of standard RNA prior to amplification were used, as indicated in the *x*-axis, whereas the corresponding cycle threshold (C_T) values are presented on the *y*-axis. Each dot represents the result of duplicate amplification of each dilution. The coefficient of determination (R^2) and the slope value (*s*) of the regression curve were calculated and are indicated.

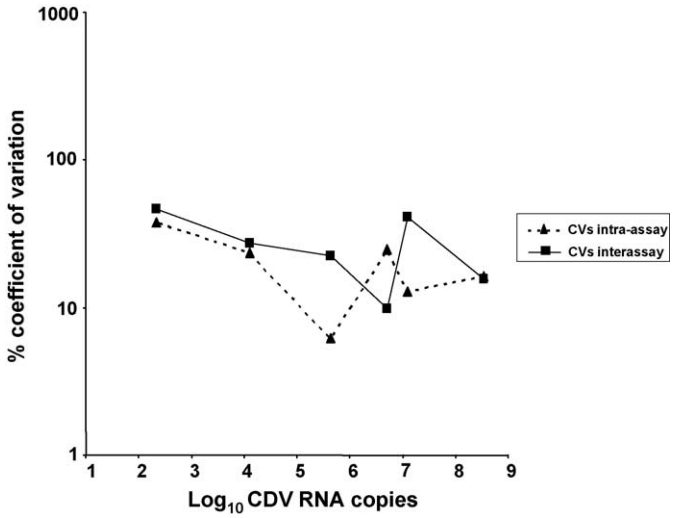

Fig. 2. Coefficient of variation intra- and inter-assay over the dynamic range of the real-time RT-PCR for canine distemper virus.

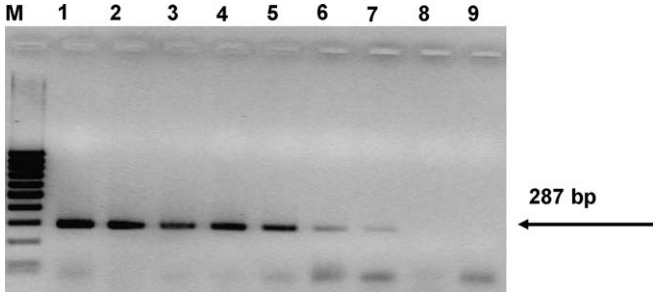

Fig. 3. Sensitivity of the conventional RT-PCR based on gene N of canine distemper virus. Lane M, molecular size marker GeneRuler 100 bp DNA Ladder (Fermentas, GmbH, St. Leon-Rot., Germany); lanes 1–9, 10-fold dilutions (undiluted to 10^{-8}) of a canine distemper virus-positive sample, containing 3.13×10^8 RNA copies/μl of template.

determine whether there is any cross-reactivity with other targets. These samples were positive controls from other assays currently being run in our lab. No cross-reactivity with CCoV type I, CCoV type II, reoviruses and rotaviruses was observed in this assay. Similarly, no detectable fluorescence signal was obtained in control negative tubes (negative samples and no-template controls), confirming that the assay was specific for the detection of canine distemper virus RNA.

To asses the reproducibility of the fluorogenic assay, the inter-assay and the intra-assay coefficient of variations (CVs) were calculated by testing in five consecutive runs (CV inter-assay) or five times in the same run (CV intra-assay) samples containing different amounts of virus RNA (Fig. 2). The intra-assay CVs were in the range of 6.19% (samples containing 4.41×10^5 RNA copies) to 37.52% (samples containing 2.18×10^2 RNA copies), whereas the inter-assay CVs were comprised between 9.79% (samples containing 6.34×10^6 RNA copies) and 46.72% (samples containing 4.09×10^2 RNA copies).

3.2. Sensitivity comparison of conventional RT-PCR and real-time RT-PCR

To assess the sensitivity, the real-time RT-PCR was compared with an RT-PCR assay used in clinical diagnostics (Frisk et al., 1999; Saito et al., 2006). A 50% homogenate containing 25 mg of tonsil sample positive to canine distemper virus (dog no. 16) was diluted 1:10 in eight steps by spiking a canine distemper virus-negative tissue homogenate. Genomic RNA was then analyzed in duplicate by both TaqMan assay and conventional RT-PCR. Real-time analysis predicted that the starting RNA copy number from the sample was 3.13×10^8 RNA copies/μl of template. The detection limit of the TaqMan assay was 1×10^2 and 3.13×10^2 copies for standard RNA and genomic RNA, respectively. Sensitivity of the conventional RT-PCR assay was as high as 3.13×10^2 copies of genomic RNA (Fig. 3). In addition, the cryolisate of a cell-culture-adapted strain, Onderstepoort, with a titre of $10^{4.00}$ $TCID_{50}$/50 μl, was diluted serially 1:10 in seven steps and assayed with both real-time and conventional PCR. Both the assays were able to detect virus RNA up to the dilution of $10^{-2.00}$ $TCID_{50}$/50 μl. Accordingly, the real-time assay and the conventional PCR were equally sensitive in the detection of virus RNA.

3.3. Internal control performance

The IC was detected in all the examined samples, with C_T values below the threshold value of 36.91. Therefore, significant RNA losses and DNA polymerase inhibition did not occur during nucleic acid extraction and PCR amplification, respectively.

3.4. Analysis of samples from dogs infected with canine distemper virus

To evaluate the suitability of the real-time RT-PCR assay for detection of canine distemper virus in clinical specimens from eight positive dogs, conjunctival swabs, urines, and blood were analyzed. As shown in Table 2, high levels of virus RNA were detected in all conjunctival swabs with a range between 5.91×10^5 and 4.93×10^7 μl of template. The virus loads in blood samples ranged between 6.26×10^4 and 7.58×10^6 RNA copies/μl of template, while in the urines reached 2.35×10^9 RNA copies/μl of template.

Quantitation of virus loads in archival brain and spleen tissues was also determined. In all the examined animals, RNA concentration ranged from 1.04×10^4 to 1.87×10^9 copies/μl of template.

Analysis of canine distemper virus distribution in dog no. 16 (Table 3) revealed high virus concentrations (3.10×10^6 to 3.13×10^8 RNA copies/μl of template) in the lymphoid tissues (tonsil, spleen, thymus, mesenteric lymph node). Interestingly, the virus was detected at high levels also in other tissues, such as skin, muscular tissues, footpad, oral mucosa, with the virus loads ranging from 2.10×10^6 to 4.25×10^8 RNA copies/μl of template.

In order to investigate the distribution of canine distemper virus throughout the brain tissues, a total of 17 samples were obtained from a positive brain. Six cortex areas and 11 medullar areas were selected, including the cerebellum. As shown in

Table 2
Analysis of samples from dogs naturally infected with canine distemper virus by real-time RT-PCR

Sample	Dog no.	Real-time titre[a]
Blood	1	7.58×10^6
Ocular swab	1	1.63×10^6
Ocular swab	2	5.33×10^6
Ocular swab	3	8.03×10^5
Ocular swab	4	4.93×10^7
Urine	4	1.95×10^3
Ocular swab	5	5.91×10^5
Blood	5	6.26×10^4
Urine	5	2.35×10^9
Ocular swab	6	1.13×10^7
Blood	7	6.08×10^5
Ocular swab	8	6.38×10^5
Urine	8	1.90×10^5
Spleen	9	4.78×10^6
Brain	9	2.80×10^7
Spleen	10	3.80×10^8
Brain	10	1.03×10^8
Spleen	11	2.54×10^7
Spleen	12	1.87×10^9
Spleen	13	1.37×10^9
Spleen	14	1.04×10^4
Spleen	15	8.77×10^4

[a] Real-time titres are expressed as number of virus RNA copies/μl of template.

Table 3
Analysis of samples collected at necroscopy from a dog positive to canine distemper virus (no. 16) by real-time RT-PCR

Tissues	Real-time titre[a]
Thymus	3.96×10^7
Tonsil	3.13×10^8
Mesenteric lymph node	2.64×10^8
Spleen	3.10×10^6
Oral swab	2.72×10^8
Mouth	1.16×10^8
Rectal swab	1.61×10^8
Nasal swab	5.71×10^6
Urines	7.03×10^7
Urinary bladder	4.41×10^4
Kidney	5.64×10^6
Heart	2.79×10^4
Liver	3.23×10^6
Gall	1.55×10^7
Testis	1.86×10^6
Cerebrospinal fluid	3.06×10^4
Lung	
Cranial lobe	1.43×10^8
Accessory lobe	6.28×10^7
Caudal lobe	1.41×10^8
Spinal cord	
Thoracic segment	5.19×10^5
Lumbar segment	1.82×10^7
Sacral segment	1.35×10^5
Bone marrow	1.91×10^6
Footpad	9.87×10^7
Skin	4.25×10^8
Muscle tissue	2.10×10^6
Blood	5.27×10^5

[a] Real-time titres are expressed as number of virus RNA copies/μl of template.

Table 4
Virus loads evidenced by real-time RT-PCR in the brain of a dog positive to canine distemper virus

Dog brain no. 16	Anatomic region	Real-time titre[a]
1	Frontal lobe	3.40×10^7
2	Frontal lobe	1.28×10^7
3	Temporal lobe	1.64×10^7
4	Parietal lobe	3.50×10^6
5	Occipital lobe	3.23×10^6
6	Cerebellar hemisphere	1.36×10^6
7	Temporal lobe	2.21×10^7
8	Prorean gyrus	4.23×10^6
9	Prorean gyrus	5.76×10^7
10	Occipital gyrus	6.64×10^5
11	Splenial gyrus	2.96×10^6
12	Hypothalamus	1.44×10^6
13	Corpus callosum: genu	7.51×10^5
14	Cingulate gyrus	1.88×10^5
15	Corpus callosum: splenium	2.17×10^2
16	Superior cerebellar vermis	7.55×10^6
17	Inferior cerebellar vermis	8.66×10^5

[a] Real-time titres are expressed as number of virus RNA copies/μl of template.

Table 4, the highest viral loads (1.64×10^7 to 5.76×10^7 RNA copies/μl of template) were detected in the frontal and temporal lobe, as well as in the prorean gyrus, while the lowest viral load was detected in the splenium of corpus callosum (2.17×10^2 RNA copies/μl of template). In the other brain samples the viral concentrations were homogeneous and ranged between 1.88×10^5 and 7.55×10^6 RNA copies/μl of template.

4. Discussion

Canine distemper is a major disease of dog, characterised by high morbidity and mortality rates, notably in dogs between 3 and 6 months of age. In recent years, several attempts have been made to improve diagnosis of canine distemper, and several conventional RT-PCR assays were developed that allow efficient virus detection in vivo from swabs or biological samples (Frisk et al., 1999; Shin et al., 1995, 2004). In addition, Seki et al. (2003) engineered VERO cells expressing the canine signaling lymphocyte activation molecule, that proved to be highly sensitive for virus isolation.

The sensitivity, specificity and rapidity of RT-PCR compared with conventional methods, including electron microscopy, virus isolation, immunofluorescence and ELISA, render this assay the first-choice diagnostic test. However, RT-PCR is technically demanding, and requires 4–8 h for a complete diagnosis with additional post-PCR analysis for the detection of PCR products.

The double-step real-time RT-PCR assay displays several advantages over conventional RT-PCR assays, increasing the laboratory throughput and enabling simultaneous processing of several samples. The technique gives exhaustive results within 3 h and the reaction is performed in a closed-tube system, not requiring additional manipulations. Although a limited carry-over contamination may occur due to separation between RT and fluorogenic PCR, a double-step assay was preferred rather

than a one-step, since one-tube methods are more expensive and less sensitive than two-step RT-PCR procedures (Nakamura et al., 1993; Decaro et al., 2004). Another major advantage of real-time RT-PCR is the ability to quantitate the viral load in clinical specimens, whereas conventional RT-PCR allows only qualitative analysis. This makes the fluorogenic assay useful for pathogenesis studies. The real-time RT-PCR developed in this study proved to be specific and no cross-amplification of non-morbillivirus RNA virus was observed. The real-time RT-PCR assay for canine distemper virus was highly reproducible and linear over a range of eight orders of magnitude, from 10^2 to 10^9 copies, allowing a precise calculation of RNA load in samples containing a wide range of viral RNA amounts. In the study, the reproducibility of the assay was high with relatively small intra- and inter-assay variability. We used the TaqMan assay to provide quantitative estimates of the virus genetic loads in a variety of tissue samples. As expected, high viral load was demonstrated in the lymphoid tissues (tonsil, spleen, mesenteric lymph nodes). High viral levels were also demonstrated in several internal organs, and, even more interestingly, in urines. This finding is consistent with what observed previously (Shen et al., 1981; Saito et al., 2006) and addresses urines as a good target for diagnosis of canine distemper virus in vivo in clinically suspected dogs.

Post-mortem diagnosis of canine distemper is usually based on detection of virus antigens by IF on brain smears. The real-time RT-PCR was used to investigate virus distribution and loads throughout the cerebral tissues in order to assess the area that is more appropriate for sampling. The complete brain of a dog was analyzed by dissection in multiple tissue samples. The frontal lobe was found to contain high viral concentrations, thus suggesting that this area is more suitable for diagnostic purposes. Additional studies are required to draw more definitive conclusions on the pattern of viral distribution and to investigate the relationships between virus localization and the occurrence of neurological signs in the different stages of infection.

The assay herewith described proved useful to shorten detection of canine distemper virus infections and allowed quantitation of viral RNA in biological samples. This will provide the basis to investigate the pathogenesis of canine distemper, with particular regards to the patterns of virus spreading and shedding. In addition, it will be helpful to evaluate the efficacy of antiviral drugs in vivo and in vitro.

References

Appel, M.J.G., 1987. Canine distemper virus. In: Appel, M.J.G. (Ed.), Virus Infections of Carnivores. Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 133–159.

Decaro, N., Pratelli, A., Campolo, M., Elia, G., Martella, V., Tempesta, M., Buonavoglia, C., 2004. Quantitation of canine coronavirus RNA in the faeces of dogs by TaqMan RT-PCR. J. Virol. Methods 119, 145–150.

Decaro, N., Martella, V., Ricci, D., Elia, G., Desario, C., Campolo, M., Cavaliere, N., Di Trani, L., Tempesta, M., Buonavoglia, C., 2005a. Genotyping-specific fluorogenic RT-PCR assays for the detection and quantitation of canine coronavirus type I and II RNA in faecal samples of dogs. J. Virol. Methods 130, 72–78.

Decaro, N., Campolo, M., Desario, C., Ricci, D., Camero, M., Lorusso, E., Elia, G., Lavazza, A., Martella, V., Buonavoglia, C., 2005b. Virological and molecular characterization of a type 3 mammalian reovirus strain isolated from a dog with diarrhea in Italy. Vet. Microbiol. 109, 19–27.

Di Trani, L., Bedini, B., Donatelli, I., Campitelli, L., Chiappini, B., De Marco, M.A., Delogu, M., Buonavoglia, C., Vaccai, G. Avian influenza virus detection by one step real-time RT-PCR assay using a 3′ minor groove binder fluorogenic probe, Biotechniques, in press.

Frisk, A.L., Konig, M., Moritz, A., Baumgartnerm, W., 1999. Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. J. Clin. Microbiol. 37 (11), 3634–3643.

Greene, C.E., Appel, M.J., 1998. Canine distemper. In: Greene, C.E. (Ed.), Infectious Diseases of the Dog and Cat, second ed. WB Saunders, Philadelphia, PA, pp. 9–22.

Jones, T.C., Hunt, R.D., King, N.W., 1997. Canine distemper. In: Jones, T.C., Hunt, R.D., King, N.W. (Eds.), Veterinary Pathology, sixth ed. Williams & Wilkins, Baltimore, pp. 311–315.

Kim, Y.H., Cho, K.W., Youn, H.Y., Yoo, H.S., Han, H.R., 2001. Detection of canine distemper virus (CDV) through one step RT-PCR combined with nested PCR. J. Vet. Sci. 2, 59–63.

Martella, V., Pratelli, A., Elia, G., Decaro, N., Tempesta, M., Buonavoglia, C., 2001. Isolation and genetic characterization of two G3P5A[3] canine rotavirus strains in Italy. J. Virol. Methods 96, 43–49.

Martella, V., Cirone, F., Elia, G., Lorusso, E., Decaro, N., Campolo, M., Desario, C., Lucente, M.S., Bellacicco, A.L., Blixenkrone-Møller, M., Carmichael, L.E., Buonavoglia, C. Heterogeneity within the hemagglutinin genes of canine distemper virus (CDV) strains detected in Italy. Vet. Microbiol., in press.

Nakamura, S., Katamine, S., Yamamoto, T., Foung, S., Kurata, T., Hirabayashi, Y., Shimada, K., Hino, S., Miyamoto, T., 1993. Amplification and detection of a single molecule of human immunodeficiency virus RNA. Virus Genes 4, 325–338.

Rzeżutka, A., Mizak, B., 2002. Application of N-PCR for diagnosis of distemper in dogs and fur animals. Vet. Microbiol. 88, 95–103.

Rima, B.K., Wishaupt, R.G., Welsh, M.J., Earle, J.A., 1995. The evolution of morbilliviruses: a comparison of nucleocapsid gene sequences including a porpoise morbillivirus. Vet. Microbiol. 44, 127–134.

Saito, T.B., Alfieri, A.A., Wosiacki, S.R., Negrão, F.J., Morais, H.S.A., Alfieri, A.F., 2006. Detection of canine distemper virus by reverse transcriptase-polymerase chain reaction in the urine of dogs with clinical signs of distemper encephalitis. Res. Vet. Sci. 80, 116–119.

Seki, F., Ono, N., Yamaguchi, R., Yanagi, Y., 2003. Efficient isolation of wild strains of canine distemper virus in Vero cells expressing canine SLAM (CD150) and their adaptability to marmoset B95a cells. J. Virol. 77, 9943–9950.

Shen, D.T., Gorham, J.R., Pedersen, V., 1981. Viruria in dogs infected with canine distemper. Vet. Med. Small Anim. Clin. 76, 1175–1177.

Shin, Y.S., Mori, T., Okita, M., Gemma, T., Kai, C., Mikami, T., 1995. Detection of canine distemper virus nucleocapsid protein gene in canine peripheral blood mononuclear cells by RT-PCR. J. Vet. Med. Sci. 57, 439–445.

Shin, Y.J., Cho, K.O., Cho, H.S., Kang, S.K., Kim, H.J., Kim, Y.H., Park, H.S., Park, N.Y., 2004. Comparison of one-step RT-PCR and a nested PCR for the detection of canine distemper virus in clinical samples. Aust. Vet. J. 82, 83–86.

von Messling, V., Harder, T.C., Moennig, V., Rautenberg, P., Nolte, I., Haas, L., 1999. Rapid and sensitive detection of immunoglobulin M (IgM) and IgG antibodies against canine distemper virus by a new recombinant nucleocapsid protein-based enzyme-linked immunosorbent assay. J. Clin. Microbiol. 37, 1049–1056.