

Use of quantitative real-time RT-PCR to investigate the correlation between viremia and viral shedding of canine distemper virus, and infection outcomes in experimentally infected dogs

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ABSTRACT. We used real-time RT-PCR and virus titration to examine canine distemper virus (CDV) kinetics in peripheral blood and rectal and nasal secretions from 12 experimentally infected dogs. Real-time RT-PCR proved extremely sensitive, and the correlation between the two methods for rectal and nasal ($r=0.78, 0.80$) samples on the peak day of viral RNA was good. Although the dogs showed diverse symptoms, viral RNA kinetics were similar; the peak of viral RNA in the symptomatic dogs was consistent with the onset of symptoms. These results indicate that real-time RT-PCR is sufficiently sensitive to monitor CDV replication in experimentally infected dogs regardless of the degree of clinical manifestation and suggest that the peak of viral RNA reflects active CDV replication.

KEY WORDS: canine distemper, kinetics, real-time RT-PCR, shedding, viremia

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Canine distemper virus (CDV; family *Paramyxoviridae*, genus *Morbillivirus*), one of the most important pathogens in dogs, causes a serious and often fatal disease. However, the clinical course and manifestations of CDV infections in dogs vary considerably. CDV replicates primarily in the lymphoid tissues of the respiratory tract. Temporary fever and lymphopenia appear at around 3–7 days post-infection (dpi) and coincide with viremia, which results in the systemic infection of most lymphoid tissues. During the acute stage, CDV is found in every secretion and excretion of the body. This phase is accompanied by various dramatic local signs, including the onset of a cutaneous rash, nasal and ocular discharge, and conjunctivitis, followed by gastrointestinal and respiratory signs [1–4, 12–16, 20, 23]. On the other hand, dogs infected experimentally with field isolates of CDV commonly show diverse clinical symptoms and mild clinical features or subclinical infections [11, 12, 15–17, 20, 23]. Thus, it is difficult to resolve and evaluate pathogenic differences among strains or to examine the protective efficacy of vaccines against field isolates. Although differences in the clinical responses of CDV-infected dogs have not been characterized, the degree of viral multiplication and spread throughout the body of infected dogs is considered a significant factor in CDV pathogenesis [11, 16–20, 23]. Therefore, a sensitive and quantitative investigation of viremia and viral shedding in infected dogs is vital to understand CDV pathogenesis.

Various methods, including viral isolation using Vero-

dogSLAMtag (Vero-DST) cell culture [22], immunofluorescence assays and reverse transcriptase-polymerase chain reaction (RT-PCR), have been applied to examine the pathogenesis of CDV [10, 11, 16, 17]. However, all of these techniques have disadvantages: viral isolation techniques are labor-intensive and time-consuming, immunofluorescence assays have limited sensitivity, and conventional RT-PCR is not quantitative. A real-time RT-PCR assay for CDV was established and shown to be highly sensitive for the quantitative detection of CDV in cell cultures and clinical specimens [8, 21]. However, there has been no report on the CDV RNA load *in vivo*. A great deal remains to be learned about changes in the CDV RNA load with time in infected dogs and the relationship between these changes and the outcome of the infection. The present study investigated the CDV RNA load in peripheral whole blood and secretions from dogs infected experimentally with CDV isolates using quantitative real-time RT-PCR and correlated these findings with clinical responses. To the best of our knowledge, the present study provides the first support for the application of quantitative real-time RT-PCR to investigate the kinetics of CDV RNA load in experimentally infected dogs. Our results indicate that real-time RT-PCR is a reliable method for monitoring the multiplication and dissemination of CDV and can be used to design future infection experiments.

CDV strain 866 was isolated using Vero cells from the peripheral mononuclear cells of a diseased dog that showed distemper with neurological signs in 1997 in Kyoto, Japan [9]. Based on a phylogenetic analysis of H gene sequences, the strain belonged to the Asia 1 group cluster. The strain was passaged five times in Vero cell cultures before use. Vero-DST and ordinary Vero cell cultures were employed for virus titration from clinical specimens and serum neutralization (SN) tests, respectively. The Vero-DST cells used in this study were kindly provided by Dr. Y. Yanagi, Department of Virology, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

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A total of 12 two-month-old specific pathogen-free beagle dogs produced in our laboratory were inoculated orally and intranasally with 1 ml each of a viral suspension of CDV strain 866 containing $10^{6.0}$ TCID₅₀/ml. The clinical manifestations, rectal temperature and body weight of each dog were recorded daily. Clinical specimens, including whole blood and rectal and nasal swabs, were collected periodically from the dogs to measure the white blood cell count and to perform real-time RT-PCR, virus titration in cell culture and SN tests. The samples used for real-time RT-PCR and virus titration were stored at -80°C prior to use. All animal procedures were performed according to the regulations and guidelines on animal ethics of Kyoto Biken Laboratories, Inc. (Kyoto, Japan) with prior approval of their Committee for Animal Experimentation.

One-step quantitative real-time RT-PCR assays were performed using previously published [21] primer and probe sets, which were designed on a highly conserved region of the P gene, to detect CDV RNA from the collected samples. The real-time PCR using these primer and probe was validated on variations among CDV strains as previously described [21]. Template RNA was prepared from 140 μl of whole blood, rectal or nasal sample using a QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA, U.S.A.) according to the manufacturer's instructions. One-step real-time RT-PCR amplification was performed using the Mx3005P Real-Time QPCR System (Stratagene, Cedar Creek, TX, U.S.A.) with a PrimeScript One-Step RT-PCR Kit (Takara Bio Inc., Otsu, Japan). Briefly, the reaction mixture for one-step real-time RT-PCR was composed of reaction master mix, 600 nM each primer (qCDVF4 and qCDVR3), 400 nM TaqMan Probe 3CDV [21] and 1 μl of sample RNA in a total volume of 25 μl . The samples were subjected to reverse transcription at 42°C for 5 min. The real-time PCR conditions consisted of one cycle of initial denaturation of 10 min at 95°C , followed by 45 cycles of 5 sec at 95°C and 30 sec at 56°C . Fluorescence data were recorded during each annealing phase. To create a standard curve, an RNA standard was prepared from an RT-PCR product amplified from the P gene of CDV strain 866 with the following primers: 5'-GAGTTCTCCTGTCTATA-ATGATAG-3' and 5'-TAGTTGACCTTTTGAGCCCA-3'. The RT-PCR product was cloned into pTA2 (Toyobo Co., Ltd., Osaka, Japan) and transcribed using an *in vitro* Transcription T7 Kit (Takara Bio Inc.). The transcript containing RNA of the P gene was purified using a deoxyribonuclease (RNase-Free DNase Set; Qiagen Inc.) and RNeasy Mini Kit (Qiagen Inc.), and quantitated using spectrophotometry (GeneQuant II RNA/DNA Calculator; GE Healthcare, Buckinghamshire, U.K.). Serial dilutions (10-fold) of the standard RNA were prepared using a dilution buffer (EASY Dilution; Takara Bio Inc.) and stored in 10- μl aliquots at -80°C until use. The linear range of quantitation of the real-time RT-PCR assay for CDV genomic RNA was determined using 10-fold serial dilutions of the standard RNA. A wide linear range, beginning at 10^2 copies and extending through 10^9 copies of standard RNA, was established. The efficiency of the standard curve result was 100.7% ($R^2=0.999$).

Virus titrations were performed in Vero-DST cell cultures.

Confluent monolayers of Vero-DST cell grown in roller tubes were inoculated with 0.1 ml of each 10-fold dilution of the rectal and nasal samples prepared in Eagle's minimum essential medium (EMEM) containing 3,000 U/ml penicillin, 300 $\mu\text{g}/\text{ml}$ streptomycin, 30 $\mu\text{g}/\text{ml}$ kanamycin and 0.6 $\mu\text{g}/\text{ml}$ amphotericin B. After adsorption at 37°C for 60 min, the cultures were washed once with EMEM. Then, 0.5 ml of fresh EMEM supplemented with 2% fetal calf serum was added. The cultures were incubated in a roller drum at 37°C for 7 days and then examined for cytopathic effects (CPEs). Cultures that showed a CPE were considered positive for CDV, and the viral contents (TCID₅₀/ml) of respective samples were calculated using the Reed-Muench method [5].

An SN test was performed using the microplate method with Vero cell cultures and Vero cell culture-adapted CDV derived from strain 866. Briefly, serial 4-fold dilutions of heat-inactivated sera were incubated at 22°C for 60 min with an equal volume of viral suspension containing 200 TCID₅₀/0.05 ml of the cell culture-adapted CDV. Four wells of Vero cell cultures were inoculated with 0.05 ml of each serum-virus mixture and incubated at 37°C in an atmosphere of 5% CO₂ for 7 days. Wells showing no CPEs were considered positive for SN antibodies, and SN antibody titers were determined using the Reed-Muench method [5].

Statistical analysis of viral RNA kinetics was performed using a one-way analysis of variance followed by multiple comparisons using Tukey's test. Pearson's coefficient of correlation (r) and the statistical significance of r were used to assess the correlation between real-time RT-PCR and virus titration in cell culture. A P -value of <0.05 was considered statistically significant.

A total of 12 dogs infected with CDV strain 866 showed diverse clinical signs of canine distemper, including anorexia, weakness, weight loss, hyperthermia, lymphopenia, diarrhea, eye discharge and/or skin rash (Table 1). One dog (no. 2), which showed relatively severe clinical signs, showed mortality symptoms 16 dpi and was euthanized. Dog no. 7 showed mild signs of a skin rash. After the onset of clinical signs in the 12 dogs, systemic signs, including anorexia, hyperthermia and lymphopenia, first appeared between 3 and 10 dpi during the relatively early stage of the experimental period. Diarrhea and peripheral signs, including an eye discharge and rash on the chin, thighs and anal area, developed after 9 dpi during the relatively late stage. Weakness and transient weight loss were observed at either or both stages in association with other signs. No respiratory or neurological signs were observed in any of the dogs.

The quantitation of CDV based on real-time RT-PCR and virus titration are shown in Fig. 1. Real-time RT-PCR for the detection of CDV from rectal and nasal secretions showed no false negative results, in that all samples positive based on virus titration in Vero-DST cells were also positive based on real-time RT-PCR. Virus titration detected viral shedding from the rectum in 12 of 12 dogs through the entire experimental period. Viral shedding from the rectum was detected in individual dogs from 4 to 14 dpi, with a mean of 2.2 days. Virus titration using nasal samples produced a positive result in 8 of 12 dogs. Viral shedding from the nose was detected in

Table 1. Clinical signs in dogs infected experimentally with CDV strain 866

Dog	Clinical signs (days post-infection)							
	Anorexia	Weakness	Weight loss ^{a)}	Hyperthermia ^{b)}	Lymphopenia ^{c)}	Diarrhea	Eye discharge	Skin rash
1	5–15	- ^{d)}	9, 10	5	8	10–14	16	-
2 ^{e)}	5–16	5, 13–16	5, 13–16	3–5, 9	-	10–14	14–16	-
3	5–15	-	9–11, 14	-	8	10–14	15, 16	-
4	6, 7, 11	-	11	6, 7, 10	8	10–12	10–13	11–13
5	6, 7, 11	-	-	4, 5, 10	-	10–12	-	12–19
6	-	10	-	9, 10	-	9–11	10, 13, 14, 20	-
7	-	-	-	-	-	-	-	10–20
8	-	-	-	-	10	-	-	19, 20
9	-	4, 5	-	4	6	19, 20	-	-
10	-	-	4	4, 5	6, 10	19, 20	-	9–20
11	-	-	-	-	6	-	-	11, 12
12	-	-	-	6	8	-	-	9–20

a) Weight loss: compared to the day before, $\geq 5\%$. b) Hyperthermia: $\geq 39.5^\circ\text{C}$. c) Lymphopenia: $< 6,000/\mu\text{l}$. d) -: absence of clinical signs. e) Dog no. 2 showed mortality symptoms at 16 days post-infection and was euthanized.

individual dogs from 6 to 16 dpi, with a mean of 1.3 days. In contrast, real-time RT-PCR detected CDV in rectal and nasal secretions in all 12 dogs, with a mean of 7.9 and 7.5 days per dog, and a range from 4 to 20 dpi and from 2 to 20 dpi, respectively. The mean viral RNA load in whole blood samples from the 12 dogs was detectable at 2 dpi and peaked at 6 dpi. The peak RNA load was significantly reduced at 12 dpi (comparison between 6 and 12 dpi, $P < 0.05$) and was not significantly reduced thereafter (comparison between 10 and 20 dpi, $P > 0.05$). The mean viral RNA load in rectal and nasal samples from the dogs was elevated from 2 dpi and peaked at 10 dpi and 10–12 dpi, respectively. Thereafter, the peak RNA load in the rectal and nasal samples was not significantly reduced until 16 and 18 dpi, respectively (comparison between 10, 16 and 18 dpi, respectively, $P > 0.05$). Correlations between real-time RT-PCR and virus titration for rectal ($r = 0.58$) and nasal ($r = 0.48$) samples from 12 dogs were poor throughout the experimental period (0–20 dpi). On the peak day of viral RNA load in individual dogs, however, the correlation between the two methods for rectal ($r = 0.78$) and nasal ($r = 0.80$) samples from the 12 dogs was good. The correlation between the two methods for rectal samples from the 12 dogs from 2 days before to 2 days after the peak day of viral RNA loads in individual dogs ($r = 0.82$) was significantly higher than the correlation between the two methods throughout the experimental period (0–20 dpi; $r = 0.58$; $P < 0.05$).

All dogs seroconverted to CDV, with SN titers ranging from 1:64 to 1:512 at 14 dpi (data not shown).

In infected dogs, real-time RT-PCR identified the CDV RNA genome in samples that were negative by virus titration using Vero-DST cells, although real-time RT-PCR (within the quantitative range) and virus titration were equally sensitive for the detection of virus culture fluid (data not shown). Thus, for CDV detection in infected dogs, real-time RT-PCR was more sensitive than virus titration. The increased sensitivity of real-time RT-PCR is likely due to the detection of RNA from incompletely packaged virus particles or viral RNA from infected epithelial cells. Scagliarini *et al.*

[21] also demonstrated that real-time PCR could be used to detect CDV RNA derived from non-infective virus in tissue culture cell lysate samples. Furthermore, the detection of viral RNA from blood samples persisted until the end of the experimental period regardless of the appearance of SN antibodies. Viremia in canine distemper is thought to occur through the spread of cell-free or leukocyte-associated virus, and neutralizing antibodies are critical to eliminate cell-free virus [4, 14]. Based on this interpretation, the detection of viral RNA in whole blood by real-time RT-PCR after the appearance of SN antibodies may represent persistent cell-associated viremia. In some cases, despite the elimination of free virus from peripheral blood, infected lymphocytes and macrophages carry virus particles to the surface epithelium of local areas, such as the alimentary and respiratory tracts [4, 12]. The consistent detection of viral RNA in rectal and nasal secretions may support the concept of a persistent virus infection in local areas.

The 12 dogs infected with CDV strain 866 in this study showed various clinical presentations. The peak viral RNA loads in the blood of the symptomatic dogs correlated with the onset of systemic signs, such as anorexia, hyperthermia and lymphopenia. The peak viral RNA loads in the local secretions of the symptomatic dogs correlated with the onset of local signs, such as diarrhea, eye discharge and skin rash. Viral RNA loads correlated well with viral titers by cell culture at the peak viral RNA loads in rectal and nasal samples. These results indicate that the peak viral RNA level quantified by real-time RT-PCR after CDV infection agreed with established active virus dynamics associated with infection outcomes. Meanwhile, some dogs did not show any signs at the peak blood and secretion levels of viral RNA. Also, there were no major differences in the loads or kinetics of CDV RNA in blood or secretions among the 12 dogs showing various clinical signs, including a relatively severe course (dog no. 2) and a mild course, such as a skin rash (dog no. 7). This indicates that real-time RT-PCR could similarly quantify CDV from infected dogs with both severe and non-severe manifestations. There may be no correlation between the

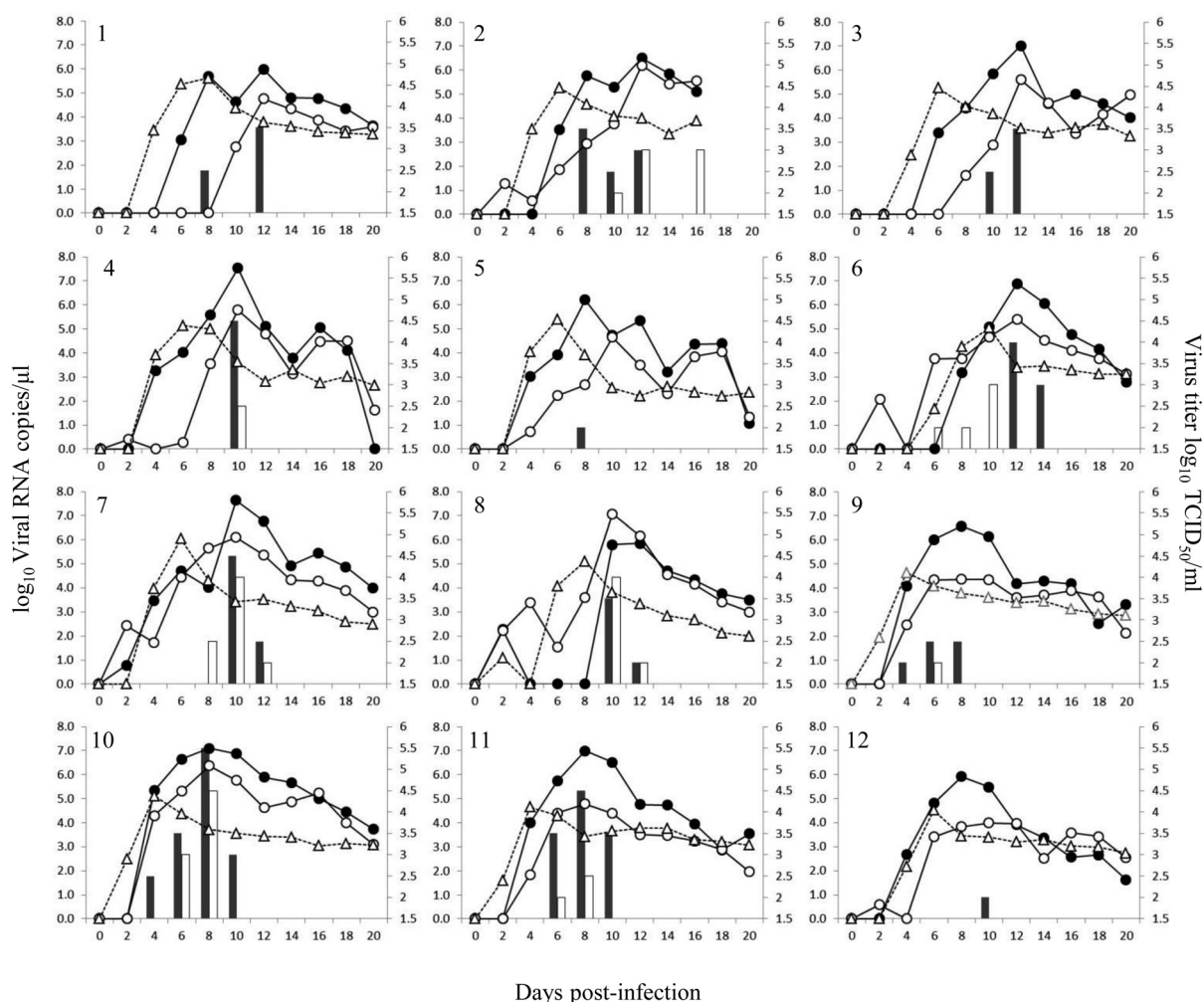


Fig. 1. The detection and quantitation of CDV in whole blood and rectal and nasal samples using real-time RT-PCR or in rectal and nasal samples using virus titration are shown for 12 individual dogs. The line and bar graphs indicate the amounts of viral RNA (copies/ μ l) and infective virus ($\text{TCID}_{50}/\text{ml}$), respectively. Closed circles and bar, rectal samples; open circles and bar, nasal samples; triangles, whole blood. The lower detection limit of quantitative real-time RT-PCR was 2.0 \log_{10} copies/ μ l.

level of viral replication in peripheral blood and epithelial cells of local areas and the development and degree of clinical manifestations. In other paramyxoviruses, it has been shown that the absence of clinical disease is not related to viral RNA shedding [7]. Based on an analysis of the molecular basis of paramyxovirus pathogenesis, virulence is influenced by the complex relationship between the virus and its host factor, so that the ability of a virus to induce disease is intimately related to the presence of proteases that are able to lyse viral fusion proteins and contribute to cell-cell fusion in infected cells [6, 24]. This host factor may explain why CDV did not always induce disease in local areas, regardless of viral replication in local epithelial cells. A further study of various CDV strains and cases of natural infection will be necessary to explain the relationship between CDV replication and clinical manifestations.

Overall, the real-time RT-PCR results reflected CDV

replication regardless of the degree of clinical manifestation in experimentally infected dogs, because real-time RT-PCR was far more sensitive than virus titration in cell culture. This study also demonstrated that the real-time RT-PCR results correlated with viral titers by cell culture at the peak of viral RNA, and the peak of viral RNA of the symptomatic dogs was consistent with the onset of symptoms. These findings suggest that the peak of viral RNA reflects active CDV replication in peripheral whole blood and secretions from dogs infected experimentally with CDV. The quantitation of CDV RNA load using this assay will be useful in comparing multiplication and dissemination among different CDV strains and to determine the protective efficacy of vaccines.

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