Protocol

Development and validation of a real-time PCR assay for specific and sensitive detection of canid herpesvirus 1

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ABSTRACT

A TaqMan-based real-time PCR assay targeting the glycoprotein B-encoding gene was developed for diagnosis of canid herpesvirus 1 (CHV-1) infection. The established assay was highly specific, since no cross-reactions were observed with other canine DNA viruses, including canine parvovirus type 2, canine minute virus, or canine adenovirus types 1 and 2. The detection limit was 10^1 and 1.20×10^1 DNA copies per $10\,\mu l^{-1}$ of template for standard DNA and a CHV-1-positive kidney sample, respectively: about 1-log higher than a gel-based PCR assay targeting the thymidine kinase gene. The assay was also reproducible, as shown by satisfactory low intra-assay and inter-assay coefficients of variation. CHV-1 isolates of different geographical origins were recognised by the TaqMan assay. Tissues and clinical samples collected from three pups which died of CHV-1 neonatal infection were also tested, displaying a wide distribution of CHV-l DNA in their organs. Unlike other CHV-1-specific diagnostic methods, this quantitative assay permits simultaneous detection and quantitation of CHV-1 DNA in a wide range of canine tissues and body fluids, thus providing a useful tool for confirmation of a clinical diagnosis, for the study of viral pathogenesis and for evaluation of the efficacy of vaccines and antiviral drugs.

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1. Introduction

Canid herpesvirus 1 (CHV-1) is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. CHV-1 host range is restricted to domestic and wild canids. Serological studies have revealed close antigenic relationships among CHV-1, felid herpesvirus 1 and phocid herpesvirus 1 (Rota and Maes, 1990). CHV-1 has a worldwide distribution and its pathogenic potential appears to be related to the age of the infected animals. Adult dogs are susceptible to CHV-1, but the infection is usually asymptomatic or subclinical, or localized to the genital tract (Decaro et al., 2008d). In contrast, primary infection or reactivation of latent infection in pregnant bitches may cause reproductive disorders, including infertility, abortion, fetal resorption or mummification, stillbirth or perinatal infection, with systemic disease and neonatal death (Decaro et al., 2008d). Recently, CHV-1 infection has also been associated with canine ocular disease (Ledbetter et al., 2006,2009a,b).

Diagnosis of CHV-1 infection usually relies on virus isolation in canine cell culture. The polymerase chain reaction (PCR) has

been established for detection of CHV-1 nucleic acid (Burr et al., 1996; Schulze and Baumgärtner, 1998; Miyoshi et al., 1999; Ronsse et al., 2005; Reubel et al., 2006). A real-time PCR assay has also been developed to monitor virus shedding in the ocular secretions after experimental reactivation of latent CHV-1 (Ledbetter et al., 2009b). However, the assay was not fully validated by assessment of analytic sensitivity, specificity, linearity and reproducibility of the method.

The development and validation of a TaqMan-based real-time PCR assay are described for rapid diagnosis of CHV-1 infection and for quantitation of the viral load in tissues and/or body fluids of infected animals.

2. Materials and methods

2.1. CHV-1 reference isolates and tissues from naturally infected pups

To validate the assay CHV reference isolates provided by other researchers and clinical samples from dogs with CHV infection were used: CHV-1 reference strains DK13, DK2 and AS-D1212 (USA) were kindly provided by Dr. L.E. Carmichael, James A. Baker Institute for Animal Health, Cornell University, Ithaca, NY. The Italian strain CHV-Mirri was kindly provided by Dr. A. Guercio, Istituto Zooprofilattico della Sicilia, Palermo, Italy. The viruses were cultivated at

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Table 1
Analysis of CHV-1 isolates and biological samples from naturally infected dogs by real-time and gel-based PCR assays.

Viral strain/Dog no.	Sample type	Real-time titre[a]	PCR
CHV-DK13	A72 cryolysate	1.51×10^8	+
CHV-DK2	A72 cryolysate	1.64×10^8	+
CHV-AS-D1212	A72 cryolysate	7.98×10^7	+
CHV-Mirri	A72 cryolysate	4.39×10^7	+
257/01-M	Vaginal swab	1.57×10^3	+
257/01-T	Femoral quadriceps muscle	3.05×10^6	+
	Mesenteric lymph node	1.03×10^7	+
	Liver	6.81×10^7	+
	Cerebrum	3.80×10^7	+
	Cerebellum	3.82×10^7	+
	Urinary bladder	5.84×10^7	+
	Lung	5.61×10^8	+
	Oesophagus	4.07×10^6	+
	Trachea	3.00×10^6	+
	Kidney	1.20×10^9	+
	Spleen	7.45×10^8	+
	Jejunum	3.54×10^8	+
	Pancreas	1.10×10^7	+
	Cardiac clot	1.98×10^7	+
	Heart	2.57×10^7	+
	Thymus	1.15×10^7	+
	Gastric content	3.02×10^6	+
	Stomach	2.99×10^6	+
	Bile	9.51×10^4	+
	Tongue	8.80×10^6	+
257/01-N	Lung	1.70×10^9	+
	Spleen	2.72×10^9	+
	Liver	3.03×10^8	+
	Kidney	2.02×10^9	+
68/04	Femoral quadriceps muscle	6.76×10^5	+
	Mesenteric lymph node	3.41×10^7	+
	Liver	3.54×10^7	+
	Cerebrum	9.75×10^6	+
	Cerebellum	7.52×10^6	+
	Urinary bladder	2.08×10^6	+
	Lung	8.45×10^7	+
	Oesophagus	3.99×10^5	+
	Trachea	7.93×10^5	+
	Kidney	5.76×10^9	+
	Spleen	3.09×10^7	+
	Jejunum	7.23×10^7	+
	Pancreas	6.20×10^6	+
	Cardiac clot	5.27×10^6	+
	Heart	1.03×10^6	+
	Thymus	8.05×10^6	+
	Gastric content	1.11×10^4	+
	Stomach	4.92×10^7	+
	Bile	2.31×10^3	+
	Tongue	2.76×10^5	+

[a] Real-time titres are expressed as number of CHV-1 DNA copies per 10 μl of template.

35 °C on canine fibroblastic A-72 cells grown in Dulbecco's modified Eagle's medium, supplemented with 10% calf fetal serum. CHV-1 isolates and clinical samples (Table 1) were collected from three newborn pups (257/01-T, 257/01-N and 68/04) which died as a result of CHV-1 systemic infection in 2001 (Decaro et al., 2002b) and in 2004 (Decaro et al., unpublished data), respectively. A vaginal swab collected from the dam of pups 257/01-T and 257/01-N was also tested.

DNA was extracted from 200 μl of viral suspension or 25 mg of tissue sample using the DNeasy Tissue Kit (Qiagen, Milan, Italy), following the manufacturer's instructions. DNA of each sample was eluted in 200 μl of AE buffer (elution buffer) and diluted 1:10 in distilled water prior to molecular analysis in order to decrease residual inhibitors of DNA polymerase activity to an ineffective concentration (Decaro et al., 2005a, 2006a,b,c).

2.2. Design of primers and TaqMan probe

In order to establish a real-time PCR assay specific for CHV-1, sequences of the glycoprotein B (gB) gene of CHV-1 strains were retrieved from GenBank and aligned using the Bioedit software package (www.mbio.ncsu.edu/BioEdit/bioedit.html). A target region useful for real-time PCR was identified by visual inspection of the sequence alignment. Primers and probe were designed using the software Beacon Designer (Bio-Rad Laboratories Srl, Milan, Italy), to amplify a 136-bp fragment of the gB gene. Primers and probe were synthesised by MWG Biotech AG (Ebersberg, Germany), with the TaqMan probe labeled with the fluorescent reporter dye 6-carboxyfluoroscein (6-FAM) at the 5′ end and with blackhole quencher 1 (BHQ1) at the 3′ end. The position and sequence of the primers and probe used for real-time PCR amplification are shown in Table 2.

2.3. Plasmid construction

For construction of CHV-1 standard DNA, the 136-bp fragment generated by the primers used for real-time PCR was cloned into a pCR® 4-TOPO® vector (TOPO TA Cloning® Kit for Sequencing, Invitrogen srl, Milan, Italy) and propagated in chemically competent *Escherichia coli* one-shot TOP10 cells, following the manufacturer's instructions. Plasmid DNA was purified from transformed cells using Wizard Plus Midiprep (Promega Italia, Milan, Italy) and quantified by spectrophotometrical analysis at 260 nm on the basis of plasmid size and of the corresponding DNA mass. Ten-fold dilutions of the plasmid, representing 10^0 to 10^9 copies of DNA/10 μl of template, were spiked into a kidney homogenate (10%, w/v), that tested negative to CHV-1-by a gel-based PCR targeting the thymidine kinase (TK) gene (Schulze and Baumgärtner, 1998). Aliquots of each dilution were frozen at −70 °C and used only once.

2.4. Real-time PCR

Real-time PCR for simultaneous detection and quantitation of CHV-1 DNA was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City CA) with iTaq™ Supermix added with ROX (Bio-Rad Laboratories Srl, Milan, Italy). The reaction mixture (25 μl) contained 12.5 μl of iTaq™ Supermix, each primer at a concentration of 600 nmol l^{-1} and the probe at a concentration of 200 nmol l^{-1}, and 10 μl of template or plasmid DNA. The thermal cycling consisted of activation of iTaq DNA polymerase at 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 45 s and annealing-extension at 60 °C for 1 min. The TaqMan assay was carried out in duplicate for each unknown and standard sample and two CHV-1-negative samples (a canine vaginal swab and a kidney sample from a dead pup) and a template control were included in each assay. The increase in the fluorescent signal was registered during the extension step of reaction and the data were analysed with the appropriate sequence detector software (7500 System Software v.1.3.1). The increase of PCR product is proportional to an exponential increase in fluorescence (ΔRn). The application software produces an amplification curve resulting from a plot of ΔRn versus cycle number. The threshold cycle number (C_T) for each sample tested was regarded as the cycle number at which the amplification curve crossed the threshold which is usually selected automatically from the average of the ΔRn of the samples between cycles 3 and 15. Lower C_T values corresponded to a greater amount of initial template and a negative result was considered to have a C_T value of 40 or more cycles.

Table 2
Sequence, position and specificity of the oligonucleotides used in the study.

Assay	Reference	Primer/probe	Sequence 5′ to 3′	Polarity	Target gene	Position[a]	Amplicon size
Real-time PCR	This study	CHV-For	ACAGAGTTGATTGATAGAAGAGGTATG	+	gB	439–465	136 bp
		CHV-Rev	CTGGTGTATTAAACTTTGAAGGCTTTA	–		548–574	
		CHV-Pb	6-FAM-TCTCTGGGGTCTTCATCCTTATCAAATGCG-BHQ1	–		510–539	
Gel-based PCR	Schulze and Baumgärtner, 1998	CHV1	TGCCGCTTTTATATAGATG	+	TK	283–301	493 bp
		CHV2	AAGCGTTGTAAAAGTTCGT	–		758–776	

[a] Oligonucleotide positions are referred to the sequence of CHV-1 glycoprotein B (GenBank accession no. AF361073) and thymidine kinase (GenBank accession no. X75765) genes for real-time and gel-based PCR amplifications, respectively.

2.5. Internal control

In order to exclude losses of DNA during the extraction step and of PCR inhibitors in the DNA extracts, an internal control (IC), consisting of ovine herpesvirus type 2 (OvHV-2) DNA (Decaro et al., 2003), was added to the lysis buffer (AL buffer, Quagen) as described previously (Decaro et al., 2005a). The fixed amount of the IC added to each sample had been calculated to give a mean C_T value in a real-time PCR assay (Hüssy et al., 2001) of 32.21 with a SD of 1.02 as calculated by 100 separate runs. Real-time PCR for IC detection was carried out in a separate run and samples in which the C_T value for the IC was >34.25 (average plus 2 SD) were excluded from the analysis.

2.6. Evaluation of specificity, sensitivity, dynamic range and reproducibility

In order to exclude cross-reactivities between CHV-1 and other canine viral pathogens, the assay was evaluated for specificity by testing DNA extracts from the following viruses: canine parvovirus types 2, 2a, 2b and 2c (Decaro et al., 2009b), canine minute virus (Decaro et al., 2002a), canine adenovirus types 1 (Decaro et al., 2007a) and 2 (Decaro et al., 2004a). Tissues samples collected from the bodies of CHV-1-uninfected pups as well as sterile water were also included in the analysis as negative controls.

To evaluate the detection limits of the real-time PCR assay, 10-fold dilutions of the plasmid DNA, ranging from 10^9 to 10^0 copies, were made in a CHV-1-negative kidney homogenate and tested subsequently. Serial 10-fold dilutions of plasmids containing from 10^1 to 10^9 copies of standard DNA and corresponding C_T values were used to plot the standard curve for quantitation of CHV-1 DNA.

The reproducibility of the assay was evaluated by testing repeatedly tissue samples containing various amounts of CHV-1 DNA, spanning the whole range covered by real-time PCR, as previously described (Decaro et al., 2005a, 2006a,b,c). Since it was not possible to collect field samples containing less than 10^2 copies of viral DNA, coefficients of variation were not calculated for 10^1 DNA copies.

2.7. Gel-based PCR

A conventional PCR assay, targeting the TK gene and based on ethidium-bromide staining, was performed for specific detection of CHV-1 DNA as described previously by Schulze and Baumgärtner (1998), with minor modifications. PCR amplification was conducted using LA PCR Kit Ver. 2.1 (TaKaRa Bio., Shiga, Japan) in a 50-μl reaction containing 1 μmol l^{-1} of primers CHV1 and CHV2 (Table 2), LA PCR Buffer (Mg^{2+} plus) 1×, 8 μl of dNTP mixture (corresponding to 400 μmol l^{-1} of each dNTP), 2.5 units of TaKaRa LA Taq^{TM} and 10 μl of 1:10 diluted template DNA. The cycling protocol consisted of pre-heating at 94 °C for 3 min following by 40 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were detected by electrophoresis in 1.5% agarose gel and visualisation under UV light after bromide ethidium staining.

To compare the sensitivity of real-time and conventional PCR, 10-fold dilutions of a kidney sample containing 1.20×10^9 copies of CHV-1 DNA were carried out on a CHV-1-negative kidney homogenate and tested subsequently by both real-time and gel-based amplifications.

3. Results

3.1. Performances of real-time PCR for CHV-1

The template controls, negative samples and other canine viruses did not produce any detectable fluorescence signal, confirming that the real-time PCR was highly specific for the detection of CHV-1 DNA.

The detection limit of the assay was estimated as 10^1 and 1.20×10^1 DNA copies per 10 μl^{-1} of template for standard DNA and for CHV-1-positive kidney sample, respectively. The gel-based PCR was slightly less sensitive: it detected 1.20×10^2 DNA copies per 10 μl^{-1} of template of the positive field sample.

Ten-fold dilutions of standard DNA were tested and used to construct a standard curve by plotting the plasmid copy number logarithm against the measured C_T values. The generated standard curve covered a linear range of nine orders of magnitude (from 10^1 to 10^9 copies of standard DNA) and showed linearity over the entire quantitation range (slope = -3.165), providing accurate measurement over a very large variety of starting target amounts.

To determine the reproducibility of the assay, intra-assay and inter-assay studies were undertaken (Fig. 1). Intra-assay CVs ranged from 8.4% (samples containing 10^8 DNA copies) to 28.6% (10^2 DNA copies), while the inter-assay CVs ranged between 15.9% (10^6 DNA copies) and 51.2% (10^2 DNA copies).

3.2. Detection of the internal control

The IC was detected in all the examined samples (the viral isolates and the clinical samples from naturally infected dogs), with C_T values below the threshold value of 34.25. Therefore, significant DNA losses did not occur during nucleic acid extraction. Also, DNA polymerase inhibition was not observed.
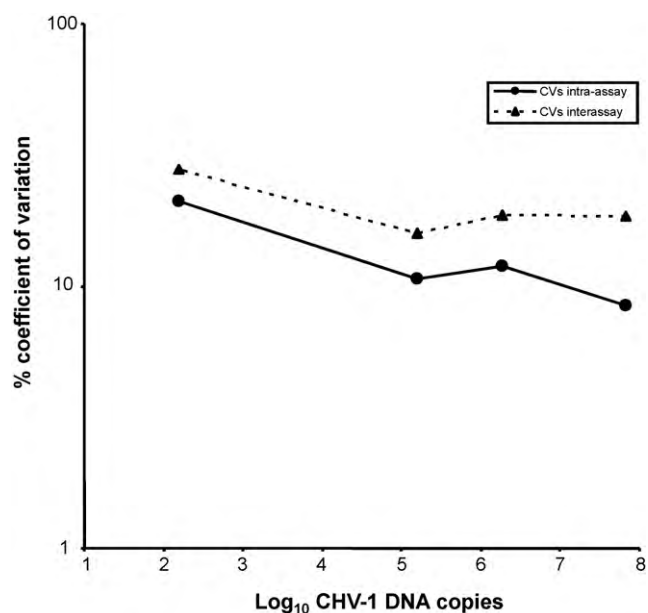
Fig. 1. Coefficients of variation intra-assay and inter-assay over the dynamic range of the CHV-1 real-time PCR assay.

3.3. Analysis of CHV-1 isolates and field samples by real-time and gel-based PCR assays

The titres of the four CHV-1 strains isolated in cell cultures ranged from 4.39×10^7 to 1.64×10^8 DNA copies/10 µl^{-1} of template (Table 1). In real-time PCR CHV-1 was detected in the vaginal swab of the dam of pups 257/01-N and 257/01-T and in all the tissues of the three infected pups. CHV-1 load was 1.57×10^3 DNA copies 10 µl^{-1} in the vaginal swab, and ranged from 2.31×10^3 to 5.76×10^9 copies 10 µl^{-1} of template in the three pups infected naturally, with the highest viral loads being detected in the kidneys. As all the samples contained high viral loads, far above the detection limit of conventional PCR, a 100% concordance was demonstrated between real-time and gel-based amplifications (Table 1).

4. Discussion

Real-time PCR assays based on the TaqMan technology have been established for the principal viruses infecting the dog, including canine parvovirus (Decaro et al., 2005a), canine enteric coronavirus (Decaro et al., 2004b), canine respiratory coronavirus (Decaro et al., 2008c; Mitchell et al., 2009), and canine distemper virus (Elia et al., 2006). Also, real-time protocols have been designed to reliably predict viral variants (Decaro et al., 2005b, 2006b), or to discriminate between vaccine and field strains (Decaro et al., 2006a,c). Precise quantitation of the viral load by real-time PCR has contributed greatly to the knowledge on viral pathogenesis (Decaro et al., 2007b, 2008a) and the efficacy of commercial vaccines (Decaro et al., 2008b, 2009a).

A real-time PCR assay has also been proposed for assessment of CHV-1 shedding by ocular secretions of dogs infected latently after treatment with corticosteroids (Ledbetter et al., 2009b). However, the analytic performance of the assay such as sensitivity, specificity, linearity and reproducibility was not evaluated thus preventing its extensive use as a standardised test in diagnostic laboratories. In addition, quantitation of CHV-1 was obtained by comparative C_T method, thus being expressed as viral load per cell and not as absolute DNA copy number, which can be obtained by processing simultaneously standard DNAs represented by 10-fold dilutions of a plasmid containing the PCR target.

The development and validation of a real-time PCR assay for detection and absolute quantitation of CHV-1 DNA in tissue samples and body fluids of dogs are described. The assay was highly sensitive and able to detect as few as 10^1 and 1.20×10^1 DNA copies 10 µl^{-1} of template for standard DNA and CHV-1-positive kidney sample, respectively, which was 1-log higher than a gel-based PCR assay targeting the thymidine kinase gene (Schulze and Baumgärtner, 1998). The precise CHV-1 DNA copy numbers (absolute quantitation) were calculated in field samples and in cell lysates of cell-propagated reference viruses by a standard curve generated by analysis of 10-fold dilutions of a plasmid DNA incorporating the target of the assay (a fragment of the gB protein gene). The intra-assay and inter-assay CVs were low. Intra-assay CV was 8.4% for the samples containing 10^8 copies of CHV-1 DNA. No cross-reactivity was observed with the DNA of other common canine viruses, showing a high specificity. In addition, a 100% concordance was demonstrated between real-time and gel-based PCR assays. Compared with the molecular methods established previously, the real-time PCR assay described above has several advantages, such as increased laboratory throughput and simultaneous processing of several samples. Compared to classical PCR protocols, the processing time required by real-time PCR is shorter, the contamination risks are lower because of the lack of post-PCR steps and the specificity is increased by the probe hybridisation. These advantages make real-time PCR an attractive method for the laboratory diagnosis of CHV-1 infection and for a precise evaluation of the extent and duration of viral shedding in animals infected naturally.

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