NOTE *Virology*

Simultaneous Detection of Canine Respiratory Disease Associated Viruses by a Multiplex Reverse Transcription-Polymerase Chain Reaction Assay

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ABSTRACT. A multiplex reverse transcription polymerase chain reaction (mRT-PCR) assay was developed for the simultaneous detection of canine distemper virus (CDV), canine respiratory coronavirus (CRCoV) and canine influenza virus (CIV). These viral pathogens are all causative agents of canine infectious respiratory disease (CIRD). The sensitivity and specificity of the mRT-PCR were determined by comparing it to a rapid antigen test (RAT) or immuno-chromatography test kit and reverse transcription-polymerase chain reaction (RT-PCR) in the detection of CDV, CRCoV and CIV antigens present in 100 clinical samples (nasal swabs and whole blood samples) from 50 dogs with respiratory disease symptoms. This study revealed that mRT-PCR had almost exactly the same performance or results were almost 100% in agreement with that of RT-PCR and RAT both in terms of the assay sensitivity and specificity which was more highly evident in detecting CIV, CDV and CRCoV antigens present in canine nasal swab samples. Therefore, this assay could be a better alternative for the definitive and simultaneous ante-mortem detection of the three viral pathogens that cause CIRD by using nasal swabs.
KEY WORDS: CDV, CIV, CRCoV, multiplex RT-PCR.

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The etiological agents involved in Canine Infectious Respiratory Disease (CIRD) are complex. They are thought to include canine distemper virus (CDV), canine influenza virus (CIV), canine respiratory coronavirus (CRCoV), canine adenovirus 2 (CAV-2), canine parainfluenza virus (CPIV), canine herpesvirus (CHV), reovirus and *Bordetella bronchiseptica* [5, 8]. CIV H3N2 strain is associated with the development of acute respiratory disease in dogs, and is a novel pathogen for CIRD in South Korea and South China [18, 25]. Serological surveys carried out in South Korea, Italy, U.K., Ireland, U.S.A. and Japan [2, 6, 7, 22, 24], have identified CRCoV as a worldwide pathogen found in the respiratory tract of dogs suffering from mild or severe respiratory disease. CDV is one of the most serious diseases in domestic dogs worldwide [11, 13, 14, 16]. It is highly contagious, affects dogs of all ages and is associated with high morbidity and mortality [10, 21]. All three viruses are present in South Korea, where 5.1% of dogs are seropositive for CIV and 12.8% are seropositive for CRCoV [2, 17]. Using one step RT-PCR, CDV has been detected in 21–70% of clinical samples such as blood, urine, nasal swabs, and saliva [3, 23].

Previous studies have shown that multiplex reverse transcription PCR (mRT-PCR) can be used to amplify more than one respiratory viral target all at the same time, thus detecting multiple respiratory infections within a single sample [4, 20, 27]. In addition, it may overcome the limitations associated with traditional diagnostic techniques, because of its increased sensitivity, specificity and rapid results. The aim of the present study was to develop a novel mRT-PCR assay for the simultaneous detection in clinical samples of three CIRD associated viruses (CIV, CRCoV and CDV), which represents a less laborious, rapid and cost-effective method in comparison to other conventional diagnostics such as viral culture and mono-specific RT-PCR. CIV H3N2 strain (A/canine/Korea/GCVP01/2007), CRCoV K39 strain and CDV Lederle strain were maintained in Madin-Darby canine kidney (MDCK) cells, human rectal tumor (HRT-18) cells and Vero cells, respectively. All cell lines were grown in Dulbecco's modified Eagle's medium (GibcoBRL, Gaithersburg, MD, U.S.A.) supplemented with 5% heat-inactivated fetal bovine serum (GibcoBRL) in a humidified 5% CO_2 atmosphere at 37°C. CIV ($10^{3.7}$ $TCID_{50}$/m*l*), CRCoV ($10^{3.2}$ $TCID_{50}$/m*l*) and CDV ($10^{3.0}$ $TCID_{50}$/m*l*) were serially diluted 2-fold to assess the sensitivity of the mRT-PCR assay. The CPIV ($10^{3.1}$ $TCID_{50}$/m*l*), CHV ($10^{3.0}$ $TCID_{50}$/m*l*) and influenza virus type B ($10^{3.6}$ $TCID_{50}$/m*l*) were used as negative controls to determine the specificity of the mRT-PCR assay. For the mRT-PCR assay, nasal swabs (n=50) and whole blood samples (n=50) were collected from 50 dogs (25 pet and 25 feral) with respiratory symptoms in 2010. The clinical samples from pet and feral dogs were supplied by an animal hospital in Jeonbuk prov-

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ince, and an animal rescue shelter in Gyeonggi province. All clinical samples were tested by mRT-PCR, RT-PCR and a rapid antigen test (RAT) that could detect both CIV (Cat. No. RG 11-07, Bionote, Hwaseong, South Korea) and CDV (Cat. No. RG 11-03, Bionote). The total viral RNA was extracted from each virus or clinical sample using the viral RNA kit (Cat. No. 52906, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. First-stand cDNA was synthesized using the first-strand synthesis system with oligo (dT) primer for RT-PCR (Cat. No. 18080–051; Invitrogen, San Diego, CA, U.S.A.). The viral DNA of CHV was extracted using Viral RNA/DNA Mini Kit (Cat. No. 12280-050; Invitrogen) according to the manufacturer's instructions. The oligonucleotide primers used to amplify the genes encoding the CIV matrix protein (Accession No: FJ560885), the CDV nucleocapsid protein (Accession No: EF4187830) and the CRCoV spike protein (Accession No: EU983107) were designed with the Clone Manager v 9.0 software (Scientific and Educational Software, Cary, NC, U.S.A.) according to the genomic sequences deposited in the GenBank database at the National Center for Biotechnology Information. The primers sequences were as follows: CIV MF 5'-TCAGGCCCCCTCAAAGCCG-3', and CIV MR 5'- CCATCGTCAACATCCACAG C-3'; CRCoV SF 5'- GCAATGCTGGTTCGGAAGAG -3', and CRCoV SR 5'- GTTGGCAT AGGTGAGCACTG -3'; and CDV NF 5'- CATGGTGGCACTCATCTTGG -3', and CDV NR 5'- TTCGGACCTCTTGT TGATGG -3'. The multiplex PCR PreMix (Cat. No. K-2111; Bioneer, Daejon, South Korea) was used for the mRT-PCR according to the manufacturer's instructions. The 20μl of mRT-PCR reaction mix contained all viral RNA/DNA and all three primer pairs for CIV, CRCoV and CDV in the same tube. The condition for mRT-PCR was follows: 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 60 sec, with a final extension step at 72°C for 5 min and holding at 4°C. The size of the amplified products, as determined by agarose gel electrophoresis was 956 bp for CIV, 442 bp for CRCoV and 624 bp for CDV (Fig. 1). Each clinical sample was tested by RT-PCR specific for each of the 3 target viruses one at a time. The primers used in RT-PCR for the detection of the CDV nucleoprotein (NP) and the CRCoV NP were PP-I and BCV-LS/NP6, respectively [9, 10]. The product sizes were 287 bp and 500 bp, respectively. Also, primers for the detection of viral genes H3 and neuramidase 2 (N2) of CIV were used in the RT-PCR assay as described previously [25]. The product sizes were 1.7 kb and 1.4 kb, respectively. After performing both RT-PCR and mRT-PCR, the PCR products were analyzed at the Zenotech Institute (Zenotech Co., Ltd., Daejon, South Korea) to confirm that the correct targets were amplified.

The sensitivity limits of the mRT-PCR assay for each of the target genes were detected at the concentration of $10^{2.5}$ $TCID_{50}$/m*l* for CIV, $10^{2.0}$ $TCID_{50}$/m*l* for CRCoV and $10^{1.8}$ $TCID_{50}$/m*l* for CDV (Fig. 2). No amplicon was detected in all the negative control viruses namely the CPIV, CHV and influenza virus type B (Fig. 1).

Comparing with the Rapid Ag kit, the mRT-PCR was

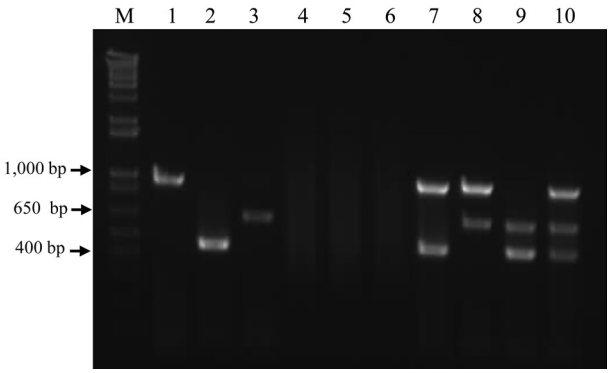

Fig. 1. Specificity of the mRT-PCR assay. M, 1 kb plus DNA ladder; lane 1: CIV ($10^{3.7}$), lane 2: CRCoV ($10^{3.2}$), lane 3: CDV ($10^{3.0}$), lane 4: CPIV ($10^{3.1}$), lane 5: CHV ($10^{3.0}$), lane 6: influenza virus type B ($10^{3.6}$), lane 7: CIV ($10^{3.7}$) + CRCoV ($10^{3.2}$), lane 8: CIV ($10^{3.7}$) + CDV ($10^{3.0}$), lane 9: CRCoV ($10^{3.2}$) + CDV ($10^{3.0}$), lane 10: CIV ($10^{3.7}$) + CRCoV ($10^{3.2}$) + CDV ($10^{3.0}$). The standard unit of virus concentration is $TCID_{50}$/m*l*.

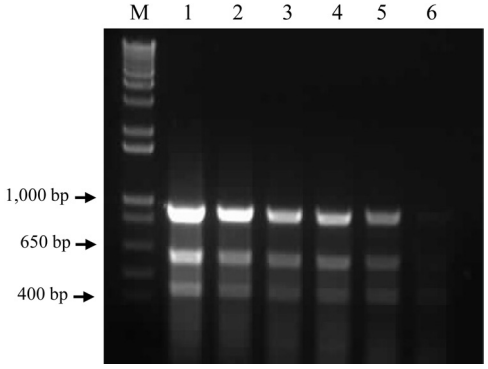

Fig. 2. Sensitivity limits of the mRT-PCR assay for each of the target genes in a mixture of viral cDNA from CIV, CRCoV, and CDV. M, 1 kb plus DNA ladder; lanes 1-6, 2-fold serial dilution for multiplex RT- PCR ($10^{3.7}$–$10^{2.2}$ $TCID_{50}$/m*l* for CIV, $10^{3.2}$–$10^{1.7}$ $TCID_{50}$/m*l* for CRCoV, $10^{3.0}$–$10^{1.5}$ $TCID_{50}$/m*l* for CDV).

100% accurate both in terms of sensitivity and specificity for CIV in both nasal swabs and whole blood samples, while for CDV it showed 97.4 and 95.1% sensitivity for nasal swab and whole blood samples, respectively, and 100% specificity for both clinical samples (Table 1). In comparison to the RT-PCRs, the mRT-PCR assay was again 100% accurate both in sensitivity and specificity for CIV and CRCoV in both nasal swabs and whole blood samples, while it showed 100% and 97.6% sensitivity to CDV in nasal swab and whole blood samples, respectively, and 100 specificity for both clinical samples (Table 2).

The mRT-PCR assay detected CIV in 8% (2/25) and 20% (5/25) of nasal swabs from pet and feral dogs, respectively. CRCoV was detected in 4% (1/25) of nasal swabs from pet dog and CDV in 76% (19/25) of nasal swabs from both pet and feral dogs. In the whole blood samples, only CDV was

Table 1. Sensitivities and specificities of the mRT-PCR assay and the with that of the Rapid Ag kit

Rapid Ag kit	mRT-PCR assay							
	CIV[a]				CDV[b]			
	NS[c]		WB[d]		NS		WB	
	+	–	+	–	+	–	+	–
+	7	0	0	0	37	0	39	0
–	0	43	0	50	1	12	2	9
Total	7	43	0	50	38	12	41	9

a) Canine influenza virus. b) Canine distemper virus. c) Nasal swab samples. d) Whole blood samples

Table 2. Sensitivities and specificities of the mRT-PCR assay and RT-PCR

RT-PCR	mRT-PCR											
	CIV[a]				CRCoV[b]				CDV[c]			
	NS[d]		WB[e]		NS		WB		NS		WB	
	+	–	+	–	+	–	+	–	+	–	+	–
+	7	0	0	0	1	0	0	0	38	0	40	0
–	0	43	0	50	0	49	0	50	0	12	1	9
Total	7	43	0	50	1	49	0	50	38	12	41	9

a) Canine influenza virus. b) Canine respiratory coronavirus. c) Canine distemper virus. d) Nasal swab samples. e) Whole blood samples.

Table 3. Detection of viruses in clinical samples using mRT-PCR

Virus	Pet dogs		Feral dogs	
	Nasal swab	Whole blood	Nasal swab	Whole blood
CIV	2/25[e]	0/25	5/25	0/25
CRCoV	1/25	0/25	0/25	0/25
CDV	19/25	20/25	19/25	21/25
CIV+CRCoV[a]	0/25	0/25	0/25	0/25
CIV+CDV[b]	1/25	0/25	3/25	0/25
CRCoV+CDV[c]	1/25	0/25	0/25	0/25
CIV+CRCoV+CDV[d]	0/25	0/25	0/25	0/25

a) Simultaneous detection of CIV and CRCoV. b) Simultaneous detection of CIV and CDV. c) Simultaneous detection of CRCoV and CDV. d) Simultaneous detection of CIV, CRCoV and CDV. e) Number of positive samples for each target virus /number of clinical samples.

detected in 80% (20/25) and 84% (21/25) of pet and feral dogs, respectively (Table 3). CDV and CIV were detected simultaneously in nasal swabs from 4% (1/25) and 12% (3/25) of pet and feral dogs, respectively, whilst co-infection with CRCoV and CDV was detected only in nasal swabs from 4% (1/25) of pet dogs (Table 3). The three viruses were confirmed by sequencing PCR products by which the correct targets were amplified. CIV positive samples were identified as H3N2 using H3 serotyping and N2 typing as described previously [25] (data not shown).

In this study, CIV was only detected in nasal swabs. This is consistent with the findings in the other studies, suggesting that CIV infected dogs only excrete the virus through the nasal discharges, and histopathological changes are observed in the upper and lower respiratory tracts [12, 25, 26]. CRCoV replicates in the upper respiratory tract and can, therefore, be detected in respiratory tissues and nasal swabs [19]. CDV can be detected in various body fluids including urine, blood, saliva, nasal secretions, and cerebrospinal fluid [15, 23]. Because all these three viruses can be detected in nasal secretions, hence nasal swabs were used for the mRT-PCR assay. In comparison to the Rapid Ag kit and RT-PCR, the mRT-PCR provided similar results for sensitivity and specificity.

Viral pathogens are responsible for a significant proportion of the CIRD burden, and new viruses are emerging worldwide. A recent study in South Korea reported CIRD in dogs caused by CIV (H3N2) and CRCoV [1, 2]. Interestingly, in this study, we indentified four dogs co-infected with CIV and CDV and, one dog co-infected with CRCoV and CDV. Therefore, there is an urgent need for an easy to perform test that can effectively identify multiple viruses simultaneously and can be used as well to screen for CIRD associated agents in canine respiratory disease samples. This study has shown

that mRT-PCR, in just one single assay, is capable to detect target genes from three viral pathogens causing CIRD. This mRT-PCR assay was developed to provide a rapid and sensitive diagnostic method for the simultaneous detection of the three viral pathogens associated with CIRD in the antemortem canine nasal swab samples. Future studies are required to expand the "coverage" of mRT-PCR enabling detection of other viral pathogens associated with CIRD

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