**FULL PAPER** *Virology*

# Development and application of multiplex PCR assays for detection of virus-induced respiratory disease complex in dogs

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ABSTRACT. Canine infectious respiratory disease complex (CIRDC) viruses have been detected in dogs with respiratory illness. Canine influenza virus (CIV), canine parainfluenza virus (CPIV), canine distemper virus (CDV), canine respiratory coronavirus (CRCoV), canine adenovirus type 2 (CAdV-2) and canine herpesvirus 1 (CaHV-1), are all associated with the CIRDC. To allow diagnosis, two conventional multiplex polymerase chain reactions (PCR) were developed to simultaneously identify four RNA and two DNA viruses associated with CIRDC. The two multiplex PCR assays were then validated on 102 respiratory samples collected from 51 dogs with respiratory illness by sensitivity and specificity determination in comparison to conventional simplex PCR and a rapid three-antigen test kit. All six viruses were detected in either individual or multiple infections. The developed multiplex PCR assays had a >87% sensitivity and 100% specificity compared to their simplex counterpart. Compared to the three-antigen test kit, the multiplex PCR assays yielded 100% sensitivity and more than 83% specificity for detection of CAdV-2 and CDV, but not for CIV. Therefore, the developed multiplex PCR modalities were able to simultaneously diagnose a panel of CIRDC viruses and facilitated specimen collection through being suitable for use of nasal or oral samples.

KEY WORDS: canine infectious respiratory disease complex (CIRDC), diagnosis, multiplex PCRs, Thailand

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Canine infectious respiratory disease complex (CIRDC), also known as kennel cough or infectious tracheobronchitis, is a highly acute respiratory disease in dogs that affects the larynx, trachea, bronchi and, occasionally, the nasal mucosa [3, 21]. The CIRDC is not only associated with infectious pathogens, but environmental factors and host immune responses also play an equally important role [7]. The pathogens causing CIRDC consist of viruses, bacteria or both, and are airborne-transmitted from infected dogs, particularly those living in poorly ventilated kennels, animal shelters and veterinary hospitals [3]. A CIRDC infection usually results in delaying of rehoming, interruption of training courses and requires high cost treatments [7]. Mildly productive cough and nasal discharge initially present as the most common clinical signs, which is self-limited within a short period in most infected dogs. It is not fatal unless other complicating factors are involved, such as secondary bacterial infection or an immunosuppressed condition [7]. Several episodes of CIRDC infection have been shown for a variety of viral agents. Canine parainfluenza virus (CPIV) is the most frequently detected agent in CIRDC dogs [16]. Canine adenovirus type 2 (CAdV-2) and canine distemper virus (CDV) have also frequently been reported in dogs with severe respiratory distress [7,16,17]. Canine herpesvirus 1 (CaHV-1) has been isolated from both puppies and adult dogs with fatal dyspnea [3, 7]. Canine influenza virus (CIV) [14, 16] and canine respiratory coronavirus (CRCoV) have recently been discovered from the respiratory tract of dogs with flu-like symptoms during a massive human flu outbreak [5, 6, 8].

Diagnosis of CIRDC-associated virus(es) is important for giving the appropriate treatment plan, prognosis and preventive strategies. Various diagnostic tests are available for these infections. However, many are not practical due to their time-consuming process, poor specificity or sensitivity, and costly diagnostic tools [10]. Thus, a rapid molecular technique is an appropriate method of choice for CIRDC virus detection. Because multiple viruses cause CIRDC, including co-infections, a multiplex polymerase chain reaction (PCR) was developed and has become commercially available as a test for respiratory tract infections [1, 10, 13, 18]. Recently, multiplex real-time PCR (qPCR) has largely replaced the conventional counterpart in order to increase the sensitivity. However, it is challenging, in terms of financial support, in developing countries and so is limited in clinical and practical uses. Thus, using a multiplex PCR would be simple, sensitive and cost-effective to screen for CIRDC viruses. Accordingly, two multiplex PCR assays were developed in this study for the simultaneous detection of CIV, CPIV, CDV, CRCoV, CAdV-2 and CaHV-1.

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Table 1. Primers used for the PCR amplification of CIRDC viruses

| Virus | Primer name | Primer sequence (5′ to 3′) | Target gene[a)] | Product size (bp) |
|---|---|---|---|---|
| CIV | CIV_M_F151 | CATGGARTGGCTAAAGACAAGACC | M | 126 |
| | CIV_M_R276 | AGGGCATTTTGGACAAAKCGTCTA | | |
| CDV | CDV_N_F768 | AACAGRRATTGCTGAGGACYTAT | NP | 290 |
| | CDV_N_R1057 | TCCARRRATAACCATGTAYGGTGC | | |
| CAdV-2 | CAdV_E3_F25073 | TATTCCAGACTCTTACCAAGAGG | E3 | 551 |
| | CAdV_E3_R25623 | ATAGACAAGGTAGTARTGYTCAG | | |
| CPIV | CPIV_N_F428 | GCCGTGGAGAGATCAATGCCTAT | NP | 187 |
| | CPIV_N_R614 | GCGCAGTCATGCACTTGCAAGT | | |
| CRCoV | CoV_16053_F | GGTTGGGAYTAYCCTAARTGTGA | S | 542 (First round PCR) |
| | CoV_16594_R | TAYTATCARAAYAATGTCTTTATGTC | | |
| | CoV_Pan_16510_R | TGATGATGGNGTTGTBTGYTATAA | | 458 (Second round PCR) |
| CaHV-1 | CaHV_GBF439 | ACAGAGTTGATTGATAGAAGAGGTATG | GB | 136 |
| | CaHV_GBR574 | CTGGTGTATTAAACTTTGAAGGCTTTA | | |

a) M=Matrix, NP=Nucleoprotein, E3=Early transcribed region, S=Spike protein, GB=Glycoprotein B.

## MATERIALS AND METHODS

*Positive control preparations*: The positive for CDV, CPIV and CAdV-2 was obtained from the modified-live vaccine Vanguard® plus 5/CV-L (Zoetis, Kalamazoo, MI, U.S.A.), containing CPIV ($10^{5.0}$ $TCID_{50}$/m*l*), CDV ($10^{2.5}$ $TCID_{50}$/m*l*) and CAdV-2 ($10^{2.9}$ $TCID_{50}$/m*l*). Meanwhile, the positive for CRCoV, CaHV-1 and CIV was derived from naturally infected dogs that were confirmed by nucleic acid sequencing. The H3N2 CIV positive was kindly provided by Prof. Alongkorn Amornsin, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University.

*Specimens*: Nasal (NS) and oropharyngeal swabs (OS) were collected from 51 suspected CIRDC suffering dogs; they were brought to veterinary hospitals residing in metropolitan Bangkok, Thailand, during February-August 2014. Those dogs that showed respiratory problems, such as nasal discharge, cough and evidence of bronchopneumonia, were included, whereas those that revealed secondary respiratory disease caused by cardiovascular and/or functional tracheal disease were excluded from the study. Vaccination status of sampled dogs was also recorded.

After taking the NS and OS using sterile rayon tipped applicators (Puritan®, Guifolrd, ME, U.S.A.), the swabs were immersed in 1% phosphate buffer saline (PBS) and kept at −80°C until assayed. The study protocol was approved by Chulalongkorn University Animal Care and Use Committee (No. 1431005).

*Viral nucleic acid extraction, quantification and reverse transcription*: Viral nucleic acid from the positive controls and specimens was extracted using the Viral Nucleic Acid Extraction Kit II (GeneAid, Taipei, Taiwan) according to manufacturer's recommendation. Nucleic acid was quantified and qualified using Nanodrop® Lite (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) at an absorbance of 260 and 280 nm to derive the $A_{260}/A_{280}$ ratio. The extracted nucleic acid was divided into two aliquots, one for reverse transcription (RT) for detection of the RNA viruses (CIV, CPIV, CDV and CRCoV) and the other for a direct PCR assay for detection of the DNA viruses (CAdV-2 and CaHV-1). The RT was performed using 100 *n*g RNA as the template for complementary DNA (cDNA) synthesis using the Omniscript® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). The cDNA and DNA were stored at −20°C until used for further PCR amplification.

*Specific primers for viruses causing CIRDC*: The sequences of the primers used for CAdV-2 (E3 gene), CDV (NP gene), CIV (M gene), CPIV (NP gene), CRCoV (S gene) and CaHV-1 (GB gene) amplification were retrieved from previous studies [4, 6, 14, 16] and are shown in Table 1. In order to ascertain the sensitivity, specificity and interaction of those primers, more than 45 sequences of each target gene were compared by multiple alignments using BioEdit Sequence Alignment Editor Version 7.1.3.0 (Ibis Biosciences, Carlsbad, CA, U.S.A.). The *in silico* specificity test was performed to select the conserved regions using BLASTn analysis in order to ensure the primer specificity without cross amplification of canine genes. Degenerate primers for CIV, CDV, CAdV-2 and CRCoV were applied (Table 1). Moreover, the canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control as reported previously [20].

*Optimization of the simplex PCR*: Prior to performing the PCR for detection of RNA viruses, a first round PCR for CRCoV was performed in order to increase the detection sensitivity. Reactions were comprised of a mixture of 2x GoTaq® Hot Start Green Master Mix (Promega, Madison, WI, U.S.A.), 0.4 $\mu$M final concentration of each outer primer (CoV_16053_F and CoV_16594_R) and 2 $\mu l$ of cDNA, and made up to 25 $\mu l$ with nuclease-free water. Reactions were performed using 3Prime G Gradient Thermal Cycle (Techne, Bristol, U.K.). Cycling conditions were comprised of an initial denaturation at 94°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The final extension was performed at 72°C for 7 min. Subsequently, the amplified CRCoV product of the first round PCR, and cDNA of the other RNA viruses (CIV, CPIV and CDV) and extracted DNA viruses (CAdV-2 and CaHV-1) were used as a template for further simplex PCR studies.

Gradient simplex PCR was performed for each virus. All reaction compositions were as mentioned above, but the gradient annealing temperature (Ta) was programmed ranging from 50°C to 59°C in order to optimize the reaction. Thermal cycling was performed with 95°C for 5 min, then 40 cycles of 95°C for 1 min, varied Ta for 1 min and 72°C for 1 min, and then finally 72°C for 10 min. The amplicons were resolved by 2% (w/v) agarose gel electrophoresis with 10% ethidium bromide in-gel staining and visualized by UV transillumination and compared to expected size of the PCR product (Table 1).

*Optimizations of multiplex PCR*: The multiplex PCR was optimized separately for RNA- and DNA-associated CIRDC viruses. The starting genetic material for RNA virus detection was derived from two compartments: (1) product from the first nested PCR of CRCoV and (2) cDNA of the other RNA viruses. Reaction composition and condition were optimized as mentioned above for the simplex PCR. The suitable Ta for all RNA and DNA viruses was selected for further comparative analysis with simplex PCR.

Sequencing of PCR amplicons was performed to confirm their correct identity and thus the specificity of the PCR reaction. Amplicons were purified with a NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) kit and submitted to The 1st BASE, Pte. Ltd. (Singapore) for direct sequencing. The derived nucleotide sequences were aligned using the BioEdit Sequence Alignment Editor version 7.0.9.0 software, and the respective consensus sequences were compared to those in the GenBank database using BLASTn analysis.

## Analysis of Specificity, Sensitivity and Reproducibility

*Specificity test*: The analytical specificity of each simplex PCR assay was evaluated by cross-reaction tests with various CIRDC-associated viruses, as well as canine parvovirus (CPV), canine enteric coronavirus (CCoV) and *Bordetella bronchiseptica.*

*Sensitivity test*: To access the analytical sensitivity of each simplex PCR assay, two-fold serial dilutions of nucleic extracted positive controls were amplified. The ten dilutions of tested controls were $2^0$ –$2^{-10}$ *n*g/ PCR reaction.

*Reproducibility*: Both intra- and inter-assay variations were measured using the positive controls and sequenced clinical samples. To assess the intra-assay variation, triplicate amplifications of the $2^{-10}$ and $2^0$ *n*g/reaction templates for the positive controls and the samples were performed in a single multiplex PCR assay. To evaluate the inter-assay variation, the above single multiplex PCR was performed as three independent multiplex PCR assays.

*Diagnostic performance of the multiplex PCR*: To evaluate the reliability of the developed multiplex PCR for clinical testing, the performance of the assay was compared to those of the simplex PCR and a commercial test kit (Antigen Rapid CIRD-3 Ag test kit, Bionote, Hwaseong, South Korea). The sensitivity, specificity, positive predictive value and negative predictive value were determined. Independent *t*-test was used to evaluate the difference between route of sample collection and number of viral detection using SPSS 22.0 (IBM Corp., New York, NY, U.S.A.)

## RESULTS

*Study population*: The 51 dogs with respiratory clinical illness included in this study were 29 males and 22 females. Most of the dogs were puppies (37.3%) or senile (23.5%). Most presented with a nasal discharge (80.4%), coughing (47.1%), loss of appetite (56.9%) and bronchopneumonia (41.2%). Only 29.4% (15/51) of dogs were vaccinated.

*Optimized and analytical performances of simplex and multiplex PCR assays*: Optimization of each simplex PCR was undertaken using positive controls and clinical samples with different cycling conditions. Different annealing temperatures were evaluated, with the optimum Ta for all virus detections being 58°C, at which temperature no primer dimers or non-specific amplicons were detected (data not shown). *In silico* and *in vitro* analytical specificity tests revealed that each primer was able to amplify the specific target DNA without any cross amplification among the CIRDC viruses, CPV, CCoV and *B. bronchiseptica*. In addition, the sequenced amplicons showed 100% sequence identity with their respective corresponding sequence in the GenBank database.

*Analytical sensitivity, specificity and reproducibility*: The sensitivity of the multiplex PCR was tested by detection of the various viruses in serial dilutions and compared with that using the simplex PCR for each particular virus. The multiplex PCR products of the tested viruses were observed at the same template dilutions as with the simplex PCR, suggesting a similar sensitivity for the simplex and multiplex PCRs (Figs. 1 and 2). The highest detection threshold was found for CDV and CRCoV, then CaHV-1 and CIV, and finally by CPIV and CAdV-2.

The specificity of the tested PCRs was evaluated by using other pathogens as mentioned above. No specific amplicons were detected in all reactions. For evaluation of the reproducibility, both intra- and inter-assay variations revealed similar results among the assays (data not shown).

*Evaluation of the multiplex PCR using clinical specimens*: The multiplex PCRs were tested on the 51 NS and 51 OS samples (Fig. 3) and compared with the simplex PCR assays for each respective virus (Table 2). The CAdV-2 and CRCoV detection had 100% sensitivity and specificity for both the NS and OS sampling sites. False negative results were observed in CaHV-1, CIV, CPIV and CDV detection when performing multiplex PCRs, which resulted in a lower sensitivity of 87.5–97.7%. The PPV (100%) of all multiplex PCRs was consistent with the specificity (100%), while the NPV (89.5–99.0%) of those reactions was contrary with their sensitivity. Neither the multiplex RT-PCR nor the multiplex PCR showed false positive results when compared with its simplex counterpart.

The comparison between the multiplex PCRs and the rapid three-antigen test kit (CAdV-2, CIV and CDV) was performed on the same samples (Table 3). With the clinical samples tested in this study, the rapid test kit yielded 100% sensitivity and a relatively high specificity for CAdV-2

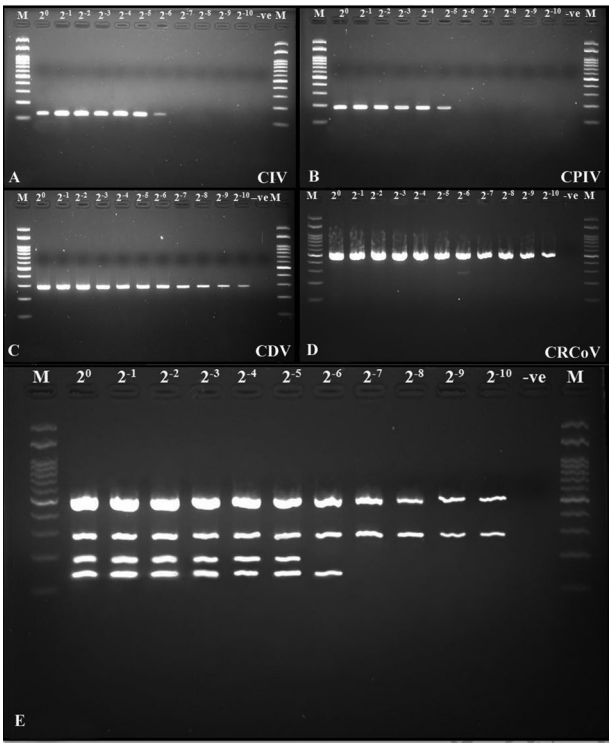


Fig. 1. Analytical sensitivity test of the (A–D) simplex and (E) multiplex RT-PCR of RNA-associated CIRDC viruses. (A) CIV, (B) CPIV, (C) CDV and (D) CRCoV. Two-fold serial dilutions of the positive controls ranging from $2^0$ $-2^{-10}$ *n*g/reaction were assayed. Detection threshold was equal in both the simplex and multiplex modalities and revealed minimal detectable dilution at $2^{-6}$(CIV), $2^{-5}$ (CPIV) and $\geq 2^{-10}$ (CDV and CRCoV) *n*g/reaction. M=DNA marker 100 bp, −ve=negative control.

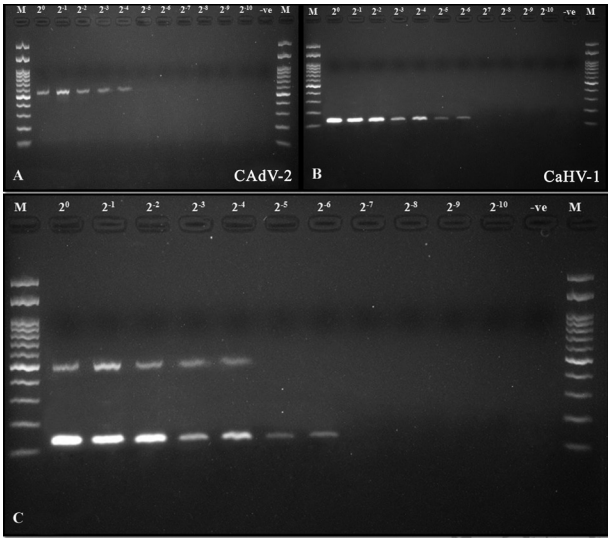


Fig. 2. Analytical sensitivity test of (A, B) simplex and (C) multiplex PCR of DNA-associated CIRDC viruses. (A) CAdV-2 and (B) CaHV-1. Two-fold serial dilutions from $2^0$ $-2^{-10}$ *n*g/reaction were tested. Detection threshold was similar in both the simplex and multiplex modalities and revealed minimal detectable dilution at $2^{-4}$ (CAdV-2) and $2^{-6}$ (CaHV-1)*n*g/reaction. M=DNA marker 100 bp, −ve=negative control.

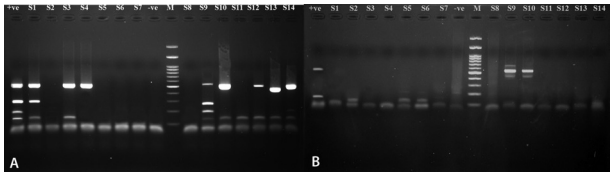


Fig. 3. Results of the (A) multiplex RT-PCR and (B) multiplex PCR tested on clinical samples (S1–S14). M=DNA marker 100 bp, −ve=negative control, +ve=positive control.

and CDV. However, for CIV, there were high numbers of PCR-positive samples detected by multiplex PCR (83/102), whereas the test kits showed negative results.

*Detection of CIRDC viruses in clinical samples by multiplex PCR*: In single infection CIV was the predominant virus detected and accounted for 23.5% (12/51) and 19.6% (10/51) positive NS and OS samples, respectively. The next most common virus was CPIV, detected at 3.9% (2/51) and 5.8% (3/51) of NS and OS samples, respectively, with 2% (1/51) being positive for CRCoV infection in both NS and OS samples. Even though the CDV, CAdV-2 and CaHV-1 were not detected as a single infection, they were detected in multiple infections in these tested samples (Table 4).

For dual infections, the most frequently detected viruses were CIV co-infected with CRCoV at 13.7% and 21.6% in NS and OS, respectively, followed by CIV with CPIV at 9.8% and 7.8% in NS and OS samples, respectively. For triple infections, CIV and CRCoV were frequently found together co-infected with other viruses, and especially with CDV and CPIV. However, one dog was negative for all tested viruses in both the NS and OS samples.

Generally, dual infections were predominant in CIRDC suffering dogs (42.2%), followed by single (28.4%) and triple (22.6%) infections. With regards to the sampling site, the frequency of positive results was not statistically different between the OS and NS sampling sites ($P$>0.05).

## DISCUSSION

The CIRDC is an important disease that impacts on dogs, especially puppies or immunosuppressed dogs, and is frequently associated with viral infections. It has gained attention recently, because many viruses have been discovered and co-infections with multiple pathogens are often fatal. Thus, the development of diagnostic tools for CIRDC-associated virus detection is necessary to enhance the diagnosis coverage. In this study, multiplex RT-PCR and multiplex PCR for the detection of CIRDC-associated RNA and DNA viruses, respectively, were developed and compared with conventional methods. Both developed multiplex PCRs could detect several viruses associated with CIRDC efficiently. The two multiplex PCRs gave similar results

Table 2. Comparison of the results from the simplex PCR and multiplex PCR for detection of CIRDC associated viruses in clinical samples

| | | Simplex PCR | | | | Total | Sensitivity | Specificity | PPV[c] | NPV[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| CAdV-2 | | CAdV-2 pos[a] | | CAdV-2 neg[a] | | | | | | |
| | | NS[b] | OS[b] | NS | OS | | | | | |
| Multiplex PCR | CAdV-2 pos | 4 | 6 | 0 | 0 | 10 | | | | |
| | CAdV-2 neg | 0 | 0 | 47 | 45 | 92 | | | | |
| | Total | 10 | | 92 | | 102 | 100 | 100 | 100 | 100 |
| CaHV-1 | | CaHV-1 pos | | CaHV-1 neg | | | | | | |
| | | NS | OS | NS | OS | | | | | |
| Multiplex PCR | CaHV-1 pos | 3 | 4 | 0 | 0 | 7 | | | | |
| | CaHV-1 neg | 1 | 0 | 47 | 47 | 95 | | | | |
| | Total | 8 | | 94 | | 102 | 87.5 | 100 | 100 | 99 |
| CIV | | CIV pos | | CIV neg | | | | | | |
| | | NS | OS | NS | OS | | | | | |
| Multiplex RT-PCR | CIV pos | 41 | 42 | 0 | 0 | 83 | | | | |
| | CIV neg | 1 | 1 | 9 | 8 | 19 | | | | |
| | Total | 85 | | 17 | | 102 | 97.7 | 100 | 100 | 89.5 |
| CPIV | | CPIV pos | | CPIV neg | | | | | | |
| | | NS | OS | NS | OS | | | | | |
| Multiplex RT-PCR | CPIV pos | 18 | 15 | 0 | 0 | 33 | | | | |
| | CPIV neg | 1 | 2 | 32 | 34 | 69 | | | | |
| | Total | 36 | | 66 | | 102 | 91.7 | 100 | 100 | 95.7 |
| CDV | | CDV pos | | CDV neg | | | | | | |
| | | NS | OS | NS | OS | | | | | |
| Multiplex RT-PCR | CDV pos | 14 | 13 | 0 | 0 | 27 | | | | |
| | CDV neg | 2 | 1 | 35 | 37 | 75 | | | | |
| | Total | 30 | | 72 | | 102 | 90 | 100 | 100 | 96 |
| CRCoV | | CRCoV pos | | CRCoV neg | | | | | | |
| | | NS | OS | NS | OS | | | | | |
| Multiplex RT-PCR | CRCoV pos | 23 | 23 | 0 | 0 | 46 | | | | |
| | CRCoV neg | 0 | 0 | 28 | 28 | 56 | | | | |
| | Total | 46 | | 56 | | 102 | 100 | 100 | 100 | 100 |

a) pos=positive, neg=negative. b) NS=nasal swab, OS=oropharyngeal swab. c) PPV=positive predictive value, NPV=negative predictive value.

equivalent to that obtained from the conventional simplex PCRs that could only detect one pathogen per reaction and so required six separate reactions per sample. Nested amplification was performed for CRCoV detection in order to increase the sensitivity of detection (Poovorawan, personal communication). Although multiplex PCR has been developed previously to detect several pathogens of CIRDC, such as CIV, CDV and CRCoV [10], its application remained limited because of the narrow range of viruses covered, with other CIRDC-associated viruses being neither detected nor ruled out. Thus, our study might provide a novel platform for whole CIRDC-virus detection.

The overall sensitivity of the multiplex RT-PCR and multiplex PCR was more than 90% and 87%, respectively, compared to their simplex counterparts. However, the detection of CRCoV was modified as a hemi-nested RT-PCR to increase its sensitivity. The false negative reactions when performing multiplex PCRs in this study might be resulted from the selection of the single optimized Ta for several primer pairs and the low amount of particular target genes [1]. These suggested for the decreased sensitivity of the developed multiplex PCRs. Moreover, there was 100% specificity in both modalities for clinical sample detection. Thus, these platforms could likely be used effectively in practice. Recently, some multiplex PCR assays were developed in order to detect the CIRDC pathogens [10]; however, the test might be immature, because only CIV, CDV and CRCoV could be detected but not for others. Thus, our study expanded the coverage of CIRDC virus detection. In an evaluation of the commercially available three-antigen rapid test kit (CAdV-2, CIV and CDV), we found only CIV detection showed an unexpected sensitivity and specificity. A previous study reported that the developed multiplex RT-PCR for H3N2 CIV, CDV and CRCoV detection had an almost 100% sensitivity and specificity compared with the conventional RT-PCR and rapid antigen test kit [10]. In contrast, our study showed that the CIV-positive samples by multiplex RT-PCR were negative when tested with the rapid antigen test kit.

Table 3. Comparison of the results from the multiplex PCR and the rapid antigen test kit for the detection of CAdV-2, CIV and CDV in clinical samples

| | | Rapid antigen test kit | | | | Total | Sensitivity | Specificity | PPV[c] | NPV[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| CAdV-2 | | CAdV-2 pos[a] | | CAdV-2 neg[a] | | | | | | |
| | | NS[b] | OS[b] | NS | OS | | | | | |
| Multiplex PCR | CAdV-2 pos | 0 | 1 | 4 | 5 | 10 | | | | |
| | CAdV-2 neg | 0 | 0 | 47 | 45 | 92 | | | | |
| | Total | 1 | | 101 | | 102 | 100 | 91.09 | 10 | 100 |
| CIV | | CIV pos | | CIV neg | | | | | | |
| | | NS | OS | NS | OS | | | | | |
| Multiplex RT-PCR | CIV pos | 0 | 0 | 41 | 42 | 83 | | | | |
| | CIV neg | 0 | 0 | 10 | 9 | 19 | | | | |
| | Total | 0 | | 102 | | 102 | UC[d] | 18.63 | 0 | 100 |
| CDV | | CDV pos | | CDV neg | | | | | | |
| | | NS | OS | NS | OS | | | | | |
| Multiplex RT-PCR | CDV pos | 6 | 6 | 8 | 7 | 27 | | | | |
| | CDV neg | 0 | 0 | 37 | 38 | 75 | | | | |
| | Total | 12 | | 90 | | 102 | 100 | 83.33 | 44.44 | 100 |

a) pos=positive, neg=negative. b) NS=nasal swab, OS=oropharyngeal swab. c) PPV=positive predictive value, NPV=negative predictive value. d)UC=unable to calculate.

This is consistent with reports that many rapid test kits might have a low sensitivity to detect the influenza virus, but could still be suitable for rapid in-house clinical applications [11, 15]. This reflects that the type of kit, viral copy number, duration of storage, route of sample collection, and type or virus strain may all influence the test results [19]. Interestingly, in this study, about 70% (71/102) of samples from the clinical respiratory illness dogs were found to have multiple infections. This finding supports that symptomatically, the CIRDC is a complex disease, which is mostly caused by co-infection with more than one pathogen. Recently, Jeoung *et al.* (2013) used both NS and whole blood samples for CIRDC virus detection, but found that only CDV (and not CIV and CRCoV) could be detected from the whole blood samples [10]. Correspondingly, respiratory swabs have been reported to be appropriate samples for the detection of respiratory pathogens [9, 16]. Thus, NS and OS served as appropriate sample sources in our study due to their ease of and non-invasive sampling nature and that they lie on the viral shedding routes. This study also suggested that the virus should be screened for in NS and OS, with detection levels at each site depending on the type of virus. The CAdV-2 and CaHV-1 mostly replicate in the lower respiratory tracts and shed via respiratory discharge, consisting with our finding that they were mostly detected in the OS, even though NS could often detect these viruses as well. However, the CAdV-2 primer pair used in this study was able to amplify CAdV-1 DNA virus which also shows airborne transmission and replicates in tonsil [3]. Therefore, the positive PCR reaction for canine adenovirus could not discriminate between CAdV-1 and CAdV-2 in this study. Additionally, CaHV-1 can be latent in various nerve ganglions, resulting in negative results from nucleic acid-based CaHV-1 detection in respiratory discharges in non-symptomatic dogs [12].

In this study, 3 out of 15 vaccinated dogs receiving, at least once, combined vaccine against CPIV, CDV and CAdV-2 showed PCR positive results for CIRDC virus detection (2 CDV positive dogs and 1 CPIV positive dog). Even though live attenuated vaccines can give false positive results with molecular testing, it is essential to discriminate between wild-type infection and recent vaccination for the prevention of false positivity in the future.

This study documented CaHV-1 and CRCoV circulation in Thailand for the first time. In 2012, CIV H3N2 was discovered in Thailand from dogs with flu-like symptoms [2]. Here, CIV and CRCoV were the most frequently detected viruses in CIRDC-infected dogs, suggesting that the viruses might spread rapidly. These viruses were not only found in single infections, but they were also found as co-infections together or with other viruses.

This study also exhibited a higher level of infections compared with a previous report [15], although this might be caused by the different timing of sample collection, population size and locations. However, it has previously been reported that infection with CRCoV and CPIV might facilitate or initiate the disease and, subsequently, enhance the entry of other pathogens [7], so the prevalence of infected dogs is then increased. Moreover, we found that the dogs that were infected with CIV, CPIV, CDV and CRCoV showed a greater severity of clinical symptoms, such as marked bronchopneumonia and sudden death (data not shown). This finding is consistent with other investigations suggesting that co-infections might augment the severity of clinical symptoms [7, 16]. Thus, advanced genetic-based detection methods, such as multiplex PCR assays, are considered as an alternative diagnostic platform for a panel of suspected CIRDC causing viruses with a high sensitivity and specificity. Because of the cost benefit and practical usage, the developed multiplex PCR assays are suitable for a screening test for disease diagnosis, quarantine and prevention measures, especially in

Table 4. CIRDC viruses detected by multiplex PCR in the 102 clinical samples from 51 dogs

| Single infection (n=29) | NS[a)] (n=15) | OS[a)] (n=14) |
|---|---|---|
| CIV | 12 | 10 |
| CPIV | 2 | 3 |
| CRCoV | 1 | 1 |
| Dual infection (n=43) | NS (n=20) | OS (n=23) |
| CIV + CPIV | 5 | 4 |
| CIV + CDV | 1 | 1 |
| CIV + CRCoV | 7 | 11 |
| CIV + CAdV-2 | 2 | 1 |
| CIV + CaHV-1 | 1 | 3 |
| CPIV + CRCoV | 1 | 0 |
| CDV + CRCoV | 3 | 2 |
| CDV + CAdV-2 | 0 | 1 |
| Triple infection (n=23) | NS (n=13) | OS (n=10) |
| CIV + CPIV + CDV | 2 | 2 |
| CIV + CPIV + CRCoV | 4 | 1 |
| CIV + CPIV + CAdV-2 | 0 | 1 |
| CIV + CDV+ CRCoV | 3 | 3 |
| CIV + CRCoV + CAdV-2 | 0 | 1 |
| CIV + CRCoV + CaHV-1 | 1 | 1 |
| CPIV + CDV + CRCoV | 1 | 0 |
| CPIV + CDV + CAdV-2 | 2 | 1 |
| 4 co-infection (n=3) | NS (n=1) | OS (n=2) |
| CIV + CPIV + CDV + CRCoV | 1 | 2 |
| 5 co-infection (n=2) | NS (n=1) | OS (n=1) |
| CIV + CPIV + CDV + CRCoV + CAdV-2 | 0 | 1 |
| CIV + CPIV + CDV + CRCoV + CaHV-1 | 1 | 0 |
| Negative (n=2) | NS (n=1) | OS (n=1) |
| | 1 | 1 |
| Total | 51 | 51 |

a) NS=nasal swab, OS=oropharyngeal swab.

developing countries.

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