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Research article

Development of a one-step multiplex (RT)-PCR for simultaneous detection of virus-induced respiratory disease complex in dogs in Vietnam

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Abstract

Canine infectious respiratory disease (CIRD) viruses, particularly canine distemper virus (CDV), canine adenovirus type 2 (CAV-2), and canine parainfluenza virus (CPIV), are the primary agents responsible for respiratory diseases in dogs. A dog can be infected with a single or multiple viruses with similar clinical signs. Although CIRD is globally prevalent in dogs, investigation into the causative viruses in Vietnam remains limited. This study aims to identify the presence of CIRD virus(es) in dogs via molecular detection. Three primer sets were newly designed and applied into (RT)-PCR reaction to detect viral genes of CDV, CAV-2, and CPIV in a commercial vaccine (Vanguard® Plus 5/L, Zoetis, USA). Both simplex and multiplex (RT)-PCR reactions using the three primer sets could detect the presence of CAV-2, CDV, and CPIV in the vaccine sample. Subsequently, the applicability of the one-step multiplex (RT)-PCR was demonstrated to test for 27 clinical nasal swab samples collected from dogs suspected of having CIRD. The results showed that our (RT)-PCR could detect CIRD virus(es) in all tested clinical samples. PCR amplicons for each representative CAV-2, CDV, and CPIV were selected for sequencing and showed high genetic similarity to respective field viruses. In conclusion, this study successfully developed a one-step multiplex (RT)-PCR reaction to detect and differentiate causative viruses of CIRD in dogs.

Keywords: CIRD, Multiplex (RT)-PCR, One-Step, Vietnam.

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INTRODUCTION

Canine infectious respiratory disease (CIRD) complex is a group of highly contagious respiratory infections in dogs caused by a combination of viruses, including canine distemper virus (CDV), canine adenovirus type 2 (CAV-2), canine parainfluenza virus (CPIV), canine influenza virus, canine herpes virus, canine respiratory coronavirus and other viruses (Erles et al., 2004; Buonavoglia and Martella, 2007). Among these pathogens, CDV, CAV-2, and CPIV have been prevalent in the CIRD in dogs worldwide (Posuwan et al., 2010; Mitchell et al., 2017). CDV belongs to the *Paramyxoviridae* family, which is the causative agent for serious respiratory disease in dogs (Appel et al., 1973). CAV-2 belongs to the *Adenoviridae* family, which primarily targets the respiratory system of dogs (Chvala et al., 2007). CPIV is categorized into the *Paramyxoviridae* family, which is widely distributed in canine populations.

The combination of CIRD viruses might cause a variety of symptoms, from mild symptoms like a persistent cough to more severe ones like pneumonia. Furthermore, multi-infections with CIRD can complicate the diagnosis because dogs with respiratory infections often show similar clinical signs such as fever, nasal discharge, sneezing, and coughing. Thus, many previous studies had been conducted to establish detection methods for CIRD viruses in dogs using multiplex/one-step multiplex (RT)-PCR in many countries such as Thailand (Posuwan et al., 2010), Korea (Jeoung et al., 2013) and China (Hao et al., 2019). Nevertheless, most of the previous multiplex PCR were established with limited purposes. For example, primer pairs used in previous multiplex (RT)-PCR were designed to target short amplicons, and that was only used for detection, not being appropriate for molecular analysis. To address this challenge, we designed specific primer pairs that can be used for simultaneous detection of the presence of CIRD viruses and for sequencing and genetic characterization of detected CIRD viruses. Therefore, our study provides an important diagnostic tool for the detection, and molecular analysis of CIRD viruses circulating in dogs in Vietnam.

MATERIALS AND METHODS

Positive control specimen

The modified-live vaccination Vanguard® Plus 5/L (Zoetis, USA) is used as a positive control for the detection of CAV-2, CDV, and CPIV in this study. The vaccine contains CDV ($10^{3.5}$ $TCID_{50}$/mL), CAV-2 ($10^{3.4}$ $TCID_{50}$/mL), and CPIV ($10^{5.5}$ $TCID_{50}$/mL).

Study areas, period, and sample collection

In 2022, a total of 27 nasal swab samples were collected from owned dogs at several veterinary clinics in Kien Giang (n=12), Ho Chi Minh (n=15), Vietnam, and were used as clinical samples. Dogs that exhibited clinical signs associated with CIRD including coughing, sneezing, nasal discharge, lethargy, fever, and respiratory distress were selected for sample collection. Prior to sample collection, none of the animals were tested using a rapid test kit to determine the presence of CIRD pathogens. Additionally, the vaccination

status of the collected dogs was unknown. Sterilized cotton swabs were used to collect the nasal swab samples, subsequently immersed in 1% phosphate buffer saline (PBS) and stored at -20 ℃ until tested.

Specific primers design

Nucleotide sequences of the H gene of CDV, E3 gene of CAV-2, and F gene of CPIV were obtained from the GenBank database used to design three new specific primer pairs for the detection of CDV, CAV-2, and CPIV via Primer-BLAST in the National Centre for Biotechnology Information (NCBI). All the primer pairs are shown in Table 1. Subsequently, the candidate primers were evaluated by the Primer3 program (https://primer3.org/) to ensure the absence of primer dimers, self-pairing, and secondary structure. Each primer pair was tested for specificity against viral DNA/RNA extracted from Vanguard Plus (Zoetis, USA) containing CAV-2, CDV, and CPIV. All specific primers used in this study were synthesized by Phu Sa Genomics, Vietnam (Table 1).

Nucleic acid extraction and simplex (RT)-PCR

DNA/RNA was extracted by TopPURE® viral DNA/RNA extraction kit (ABT, Vietnam) according to the manufacturer's instructions. The extracted DNA/RNAs were divided into two aliquots, one for simplex (RT)-PCR technique and the other for one-step multiplex (RT)-PCR technique. The RNA extracted from the positive controls and clinical samples (CDV, CPIV) was reverse transcribed to cDNA by SensiFast™ cDNA synthesis kit (Bioline, UK). Then, DNA/cDNA were used as templates for simplex (RT)-PCR technique by MyTaq™ Master Mix (Bioline, UK) in a total volume of 20 µL including 5 µL nuclease-free water, 10 µL Bio-Master mix, 1 µL forward primer (10 mM), 1 µL reverse primer (10 mM), and 3 µL template DNA/cDNA. The simplex (RT)-PCR reactions were performed in a thermal cycler under the following conditions: initial denaturation of 94 ℃ for 3 min, followed by 35 cycles of denaturation at 94 ℃ for 45 seconds, annealing at 55 ℃ for 45 seconds, extension at 72 ℃ for 1 min 30 seconds, and final extension at 72 ℃ for 10 min.

One-step multiplex (RT)-PCR

The extracted DNA/RNA were subsequently used as templates for one-step multiplex RT-PCR reaction by AccessQuick™ RT-PCR system kit (Promega, USA). Briefly, the reaction mixtures including 4 µL nuclease-free water, 10 µL AccessQuick™ master mix, 1 µL of mix forward primer (10 mM), 1 µL of mix reverse primer (10 mM), 1 µL AMV reverse transcriptase, 3 µL template DNA/RNA. The reactions were performed in a thermal cycler under the following conditions, incubate at 45 ℃ for 45 min, initial denaturation of 94 ℃ for 3 min, followed by 35 cycles of denaturation at 94 ℃ for 45 seconds, annealing at 55 ℃ for 45 seconds, extension at 72 ℃ for 1 min 30 seconds, and final extension at 72 ℃ for 10 min. Then, the PCR products were electrophoresed using 1,3% agarose gel and then visualized under UV transillumination and compared to the expected size of the PCR product (Table 1).

Nucleotide sequencing and phylogenetic analysis

For nucleotide sequencing, four PCR products of interest including one CAV-2 strain, one CPIV strain, and two CDV strains were purified by using TopPURE® ADN purification kit (TBR, Vietnam) according to the manufacturer's recommendation. The purified DNA products were subjected to nucleotide sequence determination by the Sanger sequencing method. The obtained sequences were confirmed by the Basic Local Alignment Search Tool (BLAST) method in NCBI.

For phylogenetic analysis, two CDV strains were selected to confirm the application of nucleotide sequencing results from designed primer pairs in our study for phylogenetic analysis. The bidirectional electropherograms of two CDV strains (one representative strain for Kien Giang and one representative strain for Ho Chi Minh) obtained from the Sanger method were manually inspected by Genetyx version 12 to exclude nucleotide ambiguity. The sequences of the CDV strains were edited and aligned with ClustalW by the BioEdit program (Thompson et al., 1994). The phylogenetic tree was evaluated by the Maximum Likelihood method present in the MEGA X with 1,000 bootstrap replications (Kumar et al., 2008).

RESULTS

Primer design and selection

Based on the reference nucleotide sequences of the E3 gene of CAV-2, the H gene of CDV, and the F gene of CPIV were obtained from the GenBank database. Primer pairs were designed using Pick Primers in NCBI and verified by Primer3 to ensure the absence of primer dimers, self-pairing, and secondary structure. The results showed that all newly designed primers in this study were selected to detect the presence of CDV, CAV-2, and CPIV in simplex and one-step multiplex (RT)-PCR with similar annealing temperatures and equivalent amplicon sizes. Our designed primers result in PCR products targeting variable regions and sufficient length that can be used for sequencing and genetic analysis of each etiological virus (Table 1).

Table 3 The specific primer pairs for PCR amplification of CIRD virus(es)

No.	Primer	Sequences (5'-3')	Target gene	Tm	Product size (bp)
1	CAV-2_135F	TGCCTTTGAAGGGTTTGATA	E3	55 °C	1,231
	CAV-2_1366R	ATGGCTAAGGGCCATTAAAA			
2	CDV_132F	CCTACTGATTGGAATCCTG	H	55 °C	734
	CDV_866R	TCACCCACTGCTATAGTACA			
3	CPIV_359F	GGAAACGATTAGGAACCAGT	F	55 °C	1,048
	CPIV_1407R	CAAGCTTGATGGTGCTATTG			

Note: No. 1: Canine adenovirus type 2; No. 2: Canine distemper virus; No.3: Canine parainfluenza virus

Detection of CIRD viruses in clinical samples

Amplification of DNA/cDNA samples via simplex (RT)-PCR yielded DNA amplicon sizes of 734, 1,048, and 1,231 (bp) indicating the presence of CDV, CAV-2, and CPIV in vaccine samples, respectively. Then, the simplex (RT)-PCR was used to detect CDV, CAV-2, and CPIV in 27 clinical samples from dogs. The results showed that the amplicons of the positive clinical samples were at equivalent sizes as those found in the positive live vaccine without any non-specific band. This demonstrated the high specificity of primers used for CIRD virus(es) detection via simplex (RT)-PCR (Figure 1, Left). Then, the viral DNA/RNA of nasal swab samples obtained from dogs with clinical symptoms were tested in one-step multiplex (RT)-PCR for CDV, CAV-2, and CPIV and showed that co-infections detected in each clinical sample via evidence as bands of different sizes (Figure 1, Right).

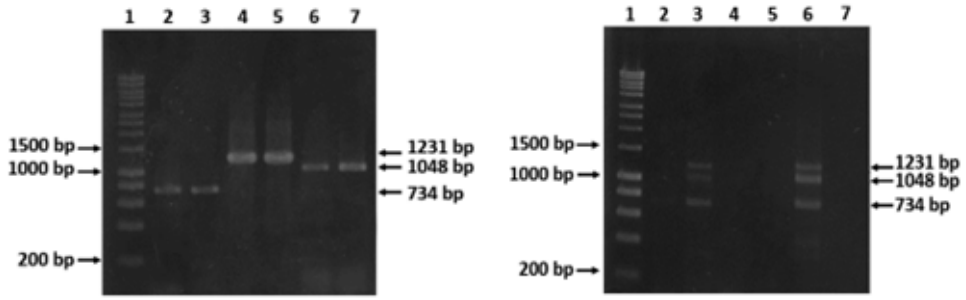

Figure 1 Detection of CIRD viruses by simplex (RT)-PCR (Left) and by one-step multiplex (RT)-PCR (Right). A fragment of 734, 1231 and 1048 bp indicate the specific band of CDV, CAV-2 and CPIV, respectively.
(Left) Lane 1: ladder 1 Kbp, Lane 2,4,6: positive control, Lane 3,5,7: field samples;(Right) Lane 1: ladder 1 Kbp, Lane 2: negative control, Lane 3: positive control, Lane 6: field sample, Lane 4,5,7: no sample.

Identification of CIRD viruses by sequencing

After (RT)-PCR amplification, 3 positive amplicons in Ho Chi Minh obtained from PCR results in clinical samples of CAV-2 (n=1), CDV (n=1), and CPIV (n=1), which were subjected to purification and confirmed via nucleotide sequencing. The positive amplicons were sequenced by the Sanger method. Then, the nucleotide sequences were identified by the BLAST analysis. The results from BLAST search showed that nucleotide sequences of most positive samples had high similarity to viral CIRD sequences previously deposited in GenBank. This sequence information was used for subsequent molecular analysis. In particular, CDV/H1, CAV-2/H1, and CPIV/H1 strains detected in Vietnam share a high identity with the VNUA CDV-Hanoi strain (Vietnam), CC-14 strain (China) and CAV2/001 (India) at about 99.85%, 99.58% and 100%, respectively (Table 2).

Table 2 The results of sequences with closest similarity from GenBank*

Isolates	Size (bp)	Homologous strain from GenBank (accession number)	Country	Identity (%)
CDV/H1	734	VNUA CDV-Hanoi4 (OM179849)	Vietnam	99,85
CPIV/H1	1048	CC-14 (KP893891)	China	99,58
CAV-2/H1	1231	CAV2/001 (MT892837)	India	100

** Results of a representative strains*

Genotyping of CDVs using phylogenetic analysis

In each sampling location, one positive sample out of 12 tested samples and one positive sample out of 15 tested samples in Kien Giang and Ho Chi Minh, respectively, were selected randomly as representative CDV samples for phylogenetic analysis. The phylogenetic tree results, based on the H gene of CDV, showed that these two representative CDVs for Ho Chi Minh (CDV/Vietnam/H1) and Kien Giang (CDV/Vietnam/KG4) belonged to two major genotypes Asia-1 and America-1, respectively. These strains were confirmed as field strains and different from vaccine CDV strains (Figure 3). In addition, the results indicate a close relationship between Vietnam CDV strains (H1, KG4, HCM/33/140816) and CDV strains isolated from China (MDJ-38, Hebei, CDV3).

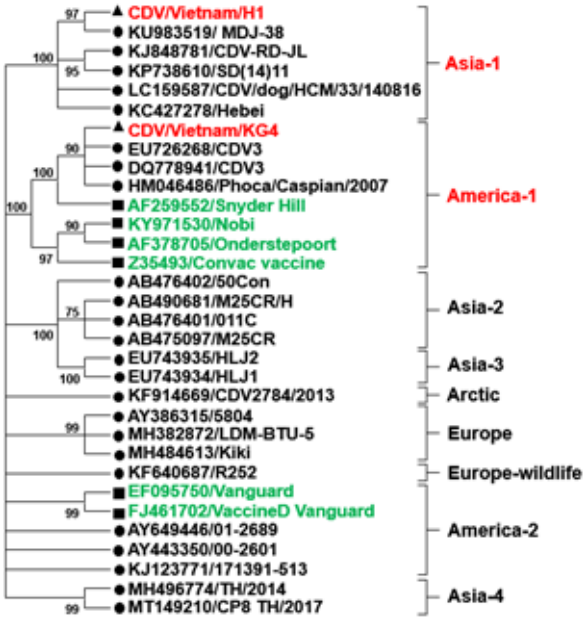

Figure 2 Phylogenetic tree based on the partial sequence of H gene of CDV in Vietnam. CDV strains detected in our study, Vaccine strains, reference CDV strains. The maximum likelihood method in MEGA X software was used to establish the phylogenetic tree (1,000 bootstrap replications).

DISCUSSION

The CIRD is a complex and important disease in dogs and is frequently associated with viral infections. Many viruses have been discovered in dogs with respiratory diseases, and co-infections might augment the severity of clinical symptoms. Therefore, the development of diagnostic methods for CIRD-associated viruses is important for providing the appropriate treatment plan, prognosis, and preventive strategies. In this study, one-step multiplex (RT)-PCR reactions were successfully developed and applied for simultaneous detection of CIRD viruses in dogs. Primer design is the first and most important step in the process of establishing a PCR reaction. According to Hao et al. (2019), primer pairs were designed in multiplex (RT)-PCR reaction must bind to conversed sequence regions, have similar annealing temperatures, and lack dimers or hairpin structures. The newly designed primer pairs in this study satisfied the above conditions. The results of PCR amplification showed that the new primer pairs designed and used in this study yielded high specificity without any cross-reaction or unexpected nonspecific amplification (Figures 1 and 2). However, the specificity and sensitivity of our detection method in this study are under investigation due to limited resources to perform standard diagnostic tests such as real-time (RT)-PCR or virus isolation. The specific primers designed for each virus can be used in a one-step multiplex (RT)-PCR reaction with a single thermal cycle. This is meaningful for further large-scale screening for CIRD viruses. Moreover, these primers yielded the desired length and obvious DNA bands, which were also sufficient for subsequent direct DNA sequencing. Recently, several multiplex/one-step multiplex PCRs were developed to detect the CIRD pathogens; however, the assays might be restricted because only some CIRD viruses could be detected, and the primer pairs were short and inappropriate for sequencing (Jeoung et al., 2013; Piewbang et al., 2016, Liu et al., 2019). Thus, our study designed primer pairs for detecting CIRD viruses in a reaction and sequencing CIRD viruses for molecular analysis. By nucleotide sequencing, the sequenced amplicons showed 99-100% sequence identity with their respective corresponding sequence in the GenBank database. The results showed that strains isolated in this study have a high similar ratio with isolates detected from Asian countries (Table 2).

The presence of CDV, CAV-2, and CPIV in clinical samples at veterinary clinics in Vietnam was detected in this study. Significantly, there have been no previous reports on CAV-2 and CPIV conducted in dogs in Vietnam. Therefore, this study represents the first identification of CAV-2 and CPIV in dogs in Vietnam. Among CIRD pathogens, CDV strains were selected for phylogenetic analysis in this study. CDVs have been categorized into many genotypes based on the H gene, and the genotypes were consistent with the geographic distribution of the viruses (Mochizuki et al., 1999; Martella et al., 2006). In this study, th CDV strain (CDV/Vietnam/H1) isolated from Ho Chi Minh belonged to genotype Asia-1, and another CDV strain (CDV/Vietnam/KG4) detected in Kien Giang belonged to genotype America-1 (Figure 3). Genotypes Asia-1 and America-1 of CDV were reported in much previous research and indicating that genotype Asia-1 is dominant among the domestic dog population in Vietnam and other Asian countries (Van Nguyen et al., 2017; Li et al., 2018; Truong et al., 2022). Significantly, genotype America-1 was also

found in our study indicating that a vaccine against CDV genotype America-1 might not completely protect domestic dogs in Vietnam against CDV infections. In addition, phylogenetic analysis revealed that all Vietnamese CDVs in this study are closely related to CDVs isolated in China (MDJ-38, Hebei, CDV3). These results suggested that there might be cross-border transmission of CDV between Vietnam and China.

CONCLUSIONS

This is the first report on the development of a one-step multiplex (RT)-PCR assay to simultaneously detect and distinguish CAV-2, CDV, and CPIV from dogs with respiratory illness in Vietnam. The primer pairs designed might be used for both detection and sequencing of the CIRD viruses. Phylogenetic results showed that the genetic diversity of CDV strains (Asia-1 and America-1) was detected in this study. Importantly, further research is necessary to expand the "coverage" of one-step multiplex (RT)-PCR enabling detection of numerous viral pathogens associated with CIRD in Vietnam.

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AUTHOR CONTRIBUTIONS

Dao Huyen Tran, Nguyen Tran Phuoc Chien, Lam Thanh Nguyen: Conceptualization and design the experiment, investigation, supervision, editing and finalization.

Nguyen Thi Lan Anh, Pham Cong Uan, To My Quyen, Nguyen Pham Nha Quan, Chau Thi Huyen Trang: Investigation, methodology, formal analysis, manuscript preparation.

CONFLICT OF INTEREST

We have no conflict of interest

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