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Short communication

# Development of a combined canine distemper virus specific RT-PCR protocol for the differentiation of infected and vaccinated animals (DIVA) and genetic characterization of the hemagglutinin gene of seven Chinese strains demonstrated in dogs

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A combined reverse-transcription polymerase chain reaction (RT-PCR) method was developed for the detection and differentiation of wild-type and vaccine strains of the canine distemper virus (CDV). A pair of primers (P1/P2) was used to detect both CDV wild-type strains and vaccines. Another pair (P3/P4) was used to detect only CDV wild-type strains. A 335 bp fragment was amplified from the genomic RNA of the vaccine and wild-type strains. A 555 bp fragment was amplified specifically from the genomic RNA of the wild-type strains. No amplification was achieved for the uninfected cells, cells infected with canine parvovirus, canine coronavirus, or canine adenovirus. The combined RT-PCR method detected effectively and differentiated the CDV wild-type and vaccine strains by two separate RT-PCRs. The method can be used for clinical detection and epidemiological surveillance. The phylogenetic analysis of the hemagglutinin gene of the local wild-type CDV strains revealed that the seven local isolates all belonged to the Asia-1 lineage, and were clustered closely with one another at the same location. These results suggested that the CDV genotype Asia-1 is circulating currently in domestic dogs in China.

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## 1. Introduction

Canine distemper virus (CDV) is a single-stranded negative RNA virus belonging to the *Morbillivirus* genus of the *Paramyxoviridae* family (Beineke et al., 2009). CDV is a highly contagious viral pathogen causing lethal systemic, nervous, and enteritis diseases in dogs and other carnivores. The CDV genome is approximately 15,690 nucleotides (nts) long and consists of 6 genes encoding nucleoprotein (NP), phosphoprotein, as well as matrix, fusion, hemagglutinin (H), and large proteins.

Due to prevention by vaccination, the outbreak of canine distemper disease has been controlled. However, the incidence of canine distemper virus (CDV) in canines and other animal populations throughout China seems to have increased in the past years. Several CDV outbreaks in vaccinated animals have been reported (Ek-Kommonen et al., 1997; Perpinan et al., 2008). Therefore, CDV wild-type and vaccine strains need to be distinguished for diagnosis and epidemiological monitoring. A previous study (Calderon et al., 2007) has shown significant differences between CDV wild-type and vaccine strains in the H protein, which displays a large number of antigenic variations. Based on this finding, several CDV differential diagnoses between wild-type and vaccine isolates have been established (Martella et al., 2007; Si et al., 2010). However, these methods are all based on reverse-transcription and nested polymerase chain reactions (RT-nPCRs); they have the disadvantages of being time-consuming and susceptible to contamination. Therefore, a method for the specific and easy detection of wild-type CDV strains is required.

In the present report, a combined reverse-transcription polymerase chain reaction (RT-PCR) method was established. The purpose was to detect and differentiate CDV wild-type and vaccine strains by two separate RT-PCRs effectively. The method provided a quick and affordable protocol for the diagnosis of CDV as well as for the differentiation of infected and vaccinated animals. This protocol can be implemented even in small laboratories. Consequently, the diagnostic possibilities for the disease are improved, accessible tools are provided to people involved in CDV eradication in endemic areas, and the CDV genetic variability as well as epidemiology can eventually be elucidated.

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## 2. Materials and methods

### 2.1. Viruses and clinical specimens

The CDV vaccine strain CDV-3 (Cheng et al., 2004) and CDV wild-type strain CDV-PS in African green monkey kidney (Vero) cell cultures were identified and preserved by the Zoonosis Lab, Institute of Special Field Economic Animal and Plant Science, the Chinese Academy of Agricultural Sciences. The two local commercial CDV vaccine products (Vacc-A and Vacc-B) were purchased at a veterinary retail shop in China and tested during the current study. The Vero cells were purchased from the China Institute of Veterinary Drug Control.

Seven CDV-positive samples were identified using RT-PCR assay, which amplified a 335 bp-long fragment of the NP gene (Wang et al., 2005). The background information on the seven CDV-positive samples collected in different locations in China is listed in Table 1.

### 2.2. RNA extraction and reverse-transcription (RT)

Total RNA was obtained from 200 mg of tissue homogenates or 200 μL of vaccine. A TRIzol® (Invitrogen) reagent was used according to the manufacturer's instructions.

RT was performed in a final volume of 20 μL containing 5 μL of RNA solutions, 4 μL of 5× RT buffer, 2 μL of deoxyribonucleotide triphosphate (10 mM each), 50 pmol of random 8-mer primers, 20 U of reverse transcriptase XL (AMV) (TaKaRa, Dalian, China), and 20 U of RNase Inhibitor. The mixture was incubated at 42 °C for 1 h, and at 70 °C for 5 min.

### 2.3. Primers and the combined two-step RT-PCR assay

The CDV cDNA products were further simultaneously amplified by a combined two-step RT-PCR assay using two primer sets to distinguish the vaccine and field strains. The specific primer sets, namely, P1/P2 (Wang et al., 2005) and P3/P4, targeted either the field isolates or the vaccine strains specifically. The primer pair P3/P4 was used to detect only wild-type CDV strains. The other pair, P1/P2, was used to detect both the wild-type and vaccine strains of CDV (Fig. 1). All primers were designed based on the genomic sequences of the CDV strains published in GenBank using the Primer Premier 5.0 software. The nt positions and sequences of the primers are shown in Table 2. The amplification conditions for the combined two-step RT-PCR assay were 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing from 52–58 °C for 1 min, DNA extension at 72 °C for 1 min, and a final extension at 72 °C for 8 min.

### 2.4. Specificity, sensitivity, and applicability tests

The two separate RT-PCRs of the combined assay were used to detect non-infected cells as well as cells infected with the CDV vaccine strain CDV-3, wild-type strain CDV-PS, canine parvovirus (CPV), canine adenovirus (CAV), and coronavirus (CCV) to test their specificities. Extracted RNA from serially diluted ($10^5$, $10^4$, $10^3$, $10^2$, $10^1$, $10^0$, $10^{-1}$, and $10^{-2}$ 50% tissue culture infective dose, $TCID_{50}$) CDV-3 strains ($10^{6.5}$ $TCID_{50}$/mL) in Vero cell cultures were assayed by RT-PCRs to determine their sensitivities. The combined RT-PCR was also performed to distinguish among the wild-type strains, field samples, vaccine strains, and commercial vaccines to validate the applicability of the test. The PCR products were resolved by 1.2% agarose gel electrophoresis.

### 2.5. Amplification, cloning, and sequencing of the H gene

The CDV cDNA products were amplified with primers reported previously by Demeter et al. (2007). The nt positions and sequences of the primer HC-1/HC-2 are shown in Table 2. The PCR reaction was subjected to 30 cycles. Each cycle consisted of denaturation at 94 °C

**Table 1**
Background of Canine Distemper virus clinical samples.

| Sample code | Location | Host | Sex | Collections | Vaccinated | Year | GenBank accession number |
|---|---|---|---|---|---|---|---|
| CDWuhan-14 | Wuhan | Dog | M | Ocular swab | Unknown | 2008 | GQ332530 |
| CDWuhan-17 | Wuhan | Dog | M | Nasal swab | Unknown | 2008 | GQ332532 |
| CDWuhan-20 | Wuhan | Dog | F | Nasal swab | Unknown | 2009 | GQ332533 |
| CDWuhan-23 | Wuhan | Dog | M | Ocular swab | Yes | 2009 | GQ332534 |
| CDWuhan-24 | Wuhan | Dog | M | Nasal swab | Yes | 2009 | GQ332535 |
| BJ-09 | Beijing | Dog | M | blood | Yes | 2010 | HQ657209 |
| Raccoon dog PS-5 | JiLin | Raccoon dog | M | liver | No | 2009 | HQ128601 |

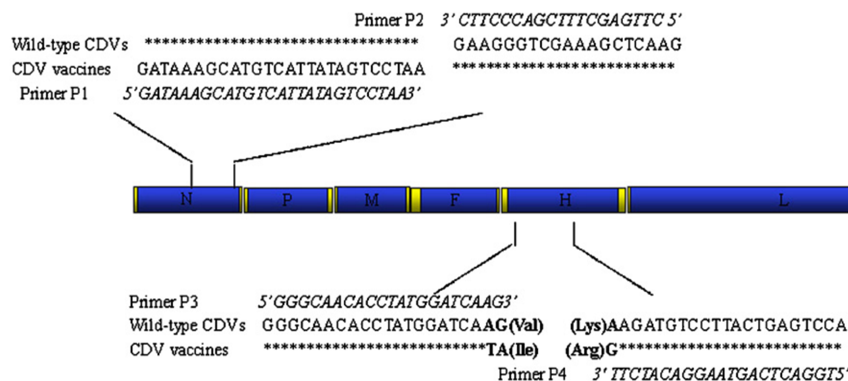


**Fig. 1.** Schematic illustration of CDV differential primers strategy by P1/P1 and P3/P4 for detecting CDV wild-type strains. The * display as the identity nucleotide. The bold letters represent 3′ terminus mismatches between CDV wild-type strain and vaccine by primers P3/P4.

**Table 2**
Primers used for RT-PCR detection and sequencing the whole H gene.

| Oligonucleotides | Nucleotide location[a] | Sequence | Amplicon |
|---|---|---|---|
| P1 | 198 | 5′GATAAAGCATGTCATTATAGTCCTAA3′ | 335bp |
| P2 | 533 | 5′CTTGAGCTTTCGACCCTTC3′ | |
| P3 | 8046 | 5′GGGCAACACCTATGGATCAAG3′ | 555bp |
| P4 | 8601 | 5′TGGACTCAGTAAGGACATCTT3′ | |
| HC-1 | 7079 | 5′AACTTAGGGCTCAGGTAGTC3′ | 2023bp |
| HC-2 | 8902 | 5′AGATGGACCTCAGGGTATAG3′ | |

[a] Location of nucleotides was based on the sequence of the complete gene of the CDV, Onderstepoort strain.

for 45 s, annealing at 53 °C for 1 min, and DNA extension at 72 °C for 2 min.

PCR products of the correct size (2023 bp long) were amplified and cloned into the pMD 18-T vector (TaKaRa, Dalian, China). For each CDV strain, 3–5 positive recombinant plasmids were sequenced in both directions using primer M13. Additional primers selected based on the obtained sequences were provided by the Shanghai Invitrogen Biotechnology Company (Shanghai, China).

### 2.6. Phylogenetic assay

Seven of the CDV field samples were selected for the amplification of the H gene of CDV by RT-PCR (Table 1). The amplified products were cloned and sequenced. The sequence data of the full-length CDV H gene were then assembled to a total length of 1824 bp using the SeqMan and EditSeq functions of the DNAStar software package. Entire H gene sequences were aligned with the sequences of other CDV H genes collected from different places around the world and available in GenBank. Subsequently, the consensus tree was edited in MEGA 4.0 (Tamura et al., 2007). A phylogenetic analysis was performed using the neighbor-joining (NJ) method, and setting the *p* distance algorithm of correction. This algorithm is expected to produce reliable phylogenies when large sets of closely related sequences are analyzed. The robustness of the phylogenetic analysis was assessed by a bootstrap analysis with 1000 replicates.

## 3. Results

### 3.1. Determination of the application conditions of the combined RT-PCR

After RNA extraction and reverse transcription, the primer pairs P1/P2 and P3/P4 were used to amplify the vaccine and wild-type strains, respectively, at different annealing temperatures. Only one specific band was observed at the annealing temperature range of 52–56.5 °C, with the most distinct band appearing at 53.5 °C. Hence, 53.5 °C was chosen for the annealing temperature in the combined RT-PCR.

### 3.2. Specificity, sensitivity, and applicability of the combined RT-PCR

A 335 bp fragment was amplified from both CDV wild-type and vaccine strains by RT-PCR with the primer pair P1/P2. On the other hand, a 555 bp fragment was detected only from the CDV wild-type strains by RT-PCR with the primer pair P3/P4 (Fig. 2A and B). By the combined RT-PCR method, amplifications were not found for the non-CDV viruses (i.e., CPV, CAV, and CCV), the uninfected cells, and the negative control of healthy animal tissues. The detection limit of the RT-PCR was $10^2$ $TCID_{50}$ for the CDV wild-type strain CDV-PS by P3/P4, and was $10^1$ $TCID_{50}$ for the vaccine strain CDV-3 by P1/P2 (Fig. 3). As illustrated in Fig. 4, all CDV commercial vaccine and field

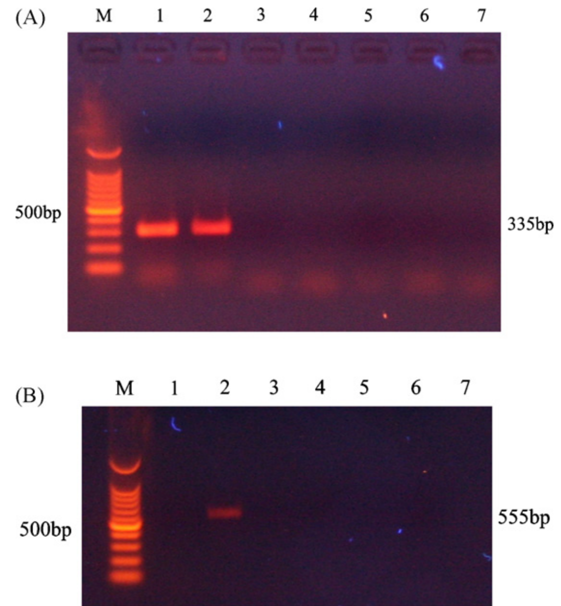


**Fig. 2.** (A) Amplification of genomes of different easily infected canine viruses by primers P1/P2 of RT-PCR reaction. M, 100 bp Ladder DNA Marker; lane 1, canine distemper virus (CDV) vaccine strain CDV3; lane 2, CDV wild-type strain CDV-PS; lane 3, canine parvovirus virus; lane 4, canine adenovirus virus; lane 5, canine coronavirus virus; lane 6, uninfected cells control; lane 7, negative control of healthy animal tissue. (B) Amplification of genomes of different easily infected canine viruses by primers P3/P4 of RT-PCR reaction. M, 100 bp Ladder DNA Marker; lane 1, canine distemper virus (CDV) vaccine strain CDV3; lane 2, CDV wild-type strain CDV-PS; lane 3, canine parvovirus virus; lane 4, canine adenovirus virus; lane 5, canine coronavirus virus; lane 6, uninfected cells control; and lane 7, negative control of healthy animal tissue.

strains were recognized by P1/P2, and yielded products that were 335 bp long. In contrast, both CDV wild-type strains yielded 555 bp products and all three CDV vaccines did not yield anything when amplified by P3/P4. After two rounds of RT-PCRs, CDV wild-type and vaccine strains were recognized. As a result, the CDV wild-type strains showed positive results (335 and 555 bp) in both RT-PCR rounds. The CDV vaccine strains showed a positive result (335 bp)

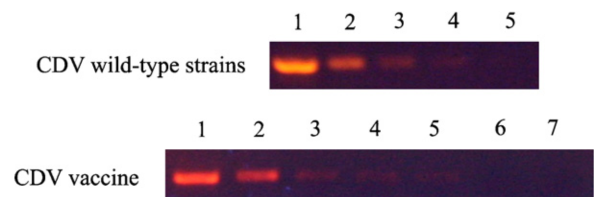


**Fig. 3.** Sensitivity of RT-PCRs to detect CDV vaccine by primers P1/P2 and CDV wild-type strain by primers P3/P4. Lanes 1–7: $10^5$ $TCID_{50}$ CDV cell culture; $10^4$ $TCID_{50}$ CDV cell culture; $10^3$ $TCID_{50}$ CDV cell culture; $10^2$ $TCID_{50}$ CDV cell culture; $10^1$ $TCID_{50}$ CDV cell culture; $10^0$ $TCID_{50}$ CDV cell culture; and $10^{-1}$ $TCID_{50}$ CDV cell culture.

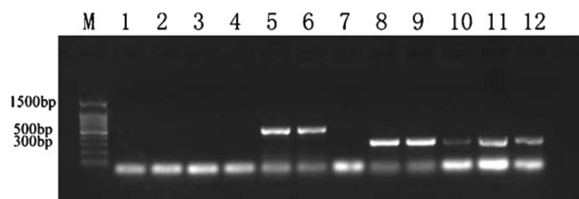


**Fig. 4.** Differential diagnosis of canine distemper virus between wide-type strains and vaccine by the combined RT-PCR test. M, 100 bp Ladder DNA Marker; lane 1 and 7: negative control without CDV template by primers P3/P4 and P1/P2, respectively; lanes 2–6: vaccine strain CDV3, Vacc-A, Vacc-B, wild-type strain CDV-PS and CDV-SD amplified from RT-PCR by primers P3/P4, respectively; lanes 8–12: vaccine strain CDV3, Vacc-A, Vacc-B, wild-type strain CDV-PS and CDV-SD amplified from RT-PCR by primers P1/P2, respectively.

in the first round and a negative result (555 bp) in the second round. The non-CDV viruses were negative in both rounds.

### *3.3. Sequencing and phylogenetic analyses*

By PCR with the primer set HC-1/HC-2, sequences of the H gene (7079–8902 nts) were identified from seven CDV local strains and one commercial vaccine (CDV3) used very commonly in China. These sequences were submitted to GenBank. The nt sequences aligned using Clustal W demonstrated that the sequence identities among the local isolates (CDV wild-type strains) ranged from 97.3% to 99.7%. On the other hand, the CDV3 vaccine strain was lower at 90.7–91.4% (Table 3). These findings indicated that the H sequence variations of CDV circulating in China and the currently used commercial vaccine are significant, as previously reported (Zhao et al., 2010).

A phylogenetic tree based on the H genes of various CDV strains was generated using the MEGA 4.0 software (Tamura et al., 2007) and the NJ method with 1000 bootstraps, as shown in Fig. 5. The strains CDwuhan-20, CDwuhan-24, CDwuhan-23, CDwuhan-14, CDwuhan-17, Raccoon dog PS-5, and BJ-09 were classified into the Asia-1 group. In general, strains from the same city or province shared a common clade. This result suggested a potential link with the phylogenetic relationship between the strains and their geographical source. All strains detected in the present study indicated a close relationship among one another as well as with strains from Taiwan (strain DogTaiwan) and Japan (strain Hamamastu). Additionally, the strain CDwuhan-17 was more distant from all other wild-type CDV strains in China, and it may eventually constitute a different genotype close to the CDV isolates in Japan (strain Yanaka).

## 4. Discussion

The primary objective of the present work was to establish an easy and quick RT-PCR method for the detection and differentiation of CDV wild-type strains and vaccines. Presently, there are several methods for CDV testing, such as virus isolation, antigen-enzyme-linked immunosorbent assay, and inmunofluorescence. However, these methods are laborious and time consuming. Recent studies have revealed that the virus isolation of CDV using Vero.DogSLAM cells can be quick and easy (Seki et al., 2003; Woma and van Vuuren, 2009; Woma et al., 2010). Nevertheless, these tests cannot differentiate between CDVs of wild-type and vaccine strains.

The CDV combined RT-PCR assay consisted of two steps of RT-PCRs. One was the traditional CDV RT-PCR assay, which detected CDV wild-type and vaccine strains. The other was the specific CDV RT-PCR assay, which only detected CDV wild-type strains. This technique, also called allele-specific oligonucleotide PCR, has originally been designed for the detection of known sequence nt polymorphisms (Chulakasian et al., 2010). Amplification discrimination mainly depended on the mismatched nt at the 3′-terminus of the primers. The PCR process only allowed amplification to occur when the most 3′-terminal nt matched its target sequences.

Comparing the genome sequences of the wild-type CDV and vaccine strains in GenBank, amino acid sequence differences were identified. The CDV wild-type strain amino acid sequence was Val and Lys at positions 331 and 502 in H protein (8067 and 8583 nts), respectively. On the other hand, the CDV vaccine strain contained Ile and Arg at the same locations. Based on the molecular sequence investigation of the wild-type local CDV strains, the H gene sequence alignment showed that the identity of the nucleic acid between local and vaccine strains ranged from 90.7% to 91.4%. The CA and G nts were also highly conserved at positions 990–991 and 1506 of the H gene in the vaccine strains. AG and A nts were at the same locations in the local wild-type strains (marked with square in Fig. 6). The CDV field strain CDWuhan-14 was not mismatched with the 3′-terminus of the P4 primer at the H gene position, whereas it had the same nt G at position 1506 in the H gene as the CDV vaccine strains. However, the reaction still continued to produce the correct fragment product. Therefore, the primer P3 may be more functional than P4. Additionally, changes in AG/CA and A/G were also observed in other Asia-1 CDVs, Asia-2 CDVs, America-2 CDVs, Europe, and Europe wildlife CDVs. In contrast, a change in GG/CA was found in Africa CDVs (Fig. 7).

Given the developments on CDV molecular research, an increasing number of CDV sequences are being deposited in GenBank. Hence, CDV molecular diagnosis methods, such as RT-PCR and real-time RT-PCR, have been significantly developed (Martella et al., 2007; Scagliarini et al., 2007). Recently, several CDV differential

**Table 3**
Comparison of the nucleotide sequences of field isolates from China with vaccine-like reference strains and commercial CDV3 vaccine using the CDV hemagglutinin gene (nucleotides 7079–8902).

| | Percentage identity | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CDwuhan-14 | CDwuhan-17 | CDwuhan-20 | CDwuhan-23 | CDwuhan-24 | PS-09 | BJ-9 | Onderstepoort | SnyderHill | Convac | CDV3 |
| CDwuhan-14 | | 97.5 | 98.4 | 99.1 | 98.5 | 97.8 | 98.0 | 90.8 | 91.1 | 91.3 | 91.2 |
| CDwuhan-17 | 2.5 | | 97.3 | 98.0 | 97.4 | 97.3 | 97.3 | 90.7 | 91.2 | 91.4 | 91.3 |
| CDwuhan-20 | 1.6 | 2.8 | | 99.0 | 99.7 | 97.5 | 97.6 | 90.5 | 90.8 | 91.0 | 91.0 |
| CDwuhan-23 | 0.9 | 2.1 | 1.0 | | 99.1 | 98.2 | 98.3 | 90.9 | 91.3 | 91.4 | 91.4 |
| CDwuhan-24 | 1.5 | 2.7 | 0.3 | 0.9 | | 97.6 | 97.8 | 90.6 | 91.0 | 91.1 | 91.1 |
| PS-5 | 2.2 | 2.8 | 2.5 | 1.8 | 2.4 | | 98.7 | 90.4 | 90.7 | 90.8 | 90.7 |
| BJ-09 | 2.0 | 2.7 | 2.4 | 1.7 | 2.3 | 1.3 | | 90.5 | 90.7 | 90.8 | 90.8 |
| Onderstepoort | 9.9 | 10.0 | 10.3 | 9.8 | 10.2 | 10.4 | 10.3 | | 96.3 | 97.9 | 96.4 |
| SnyderHill | 9.6 | 9.5 | 9.9 | 9.4 | 9.8 | 10.1 | 10.1 | 3.8 | | 97.1 | 98.8 |
| Convac | 9.4 | 9.3 | 9.8 | 9.3 | 9.6 | 9.9 | 9.9 | 2.1 | 3.0 | | 97.1 |
| CDV3 | 9.4 | 9.4 | 9.7 | 9.2 | 9.6 | 10.0 | 9.9 | 3.7 | 1.2 | 2.9 | |
| | | | | | Divergence | | | | | | |

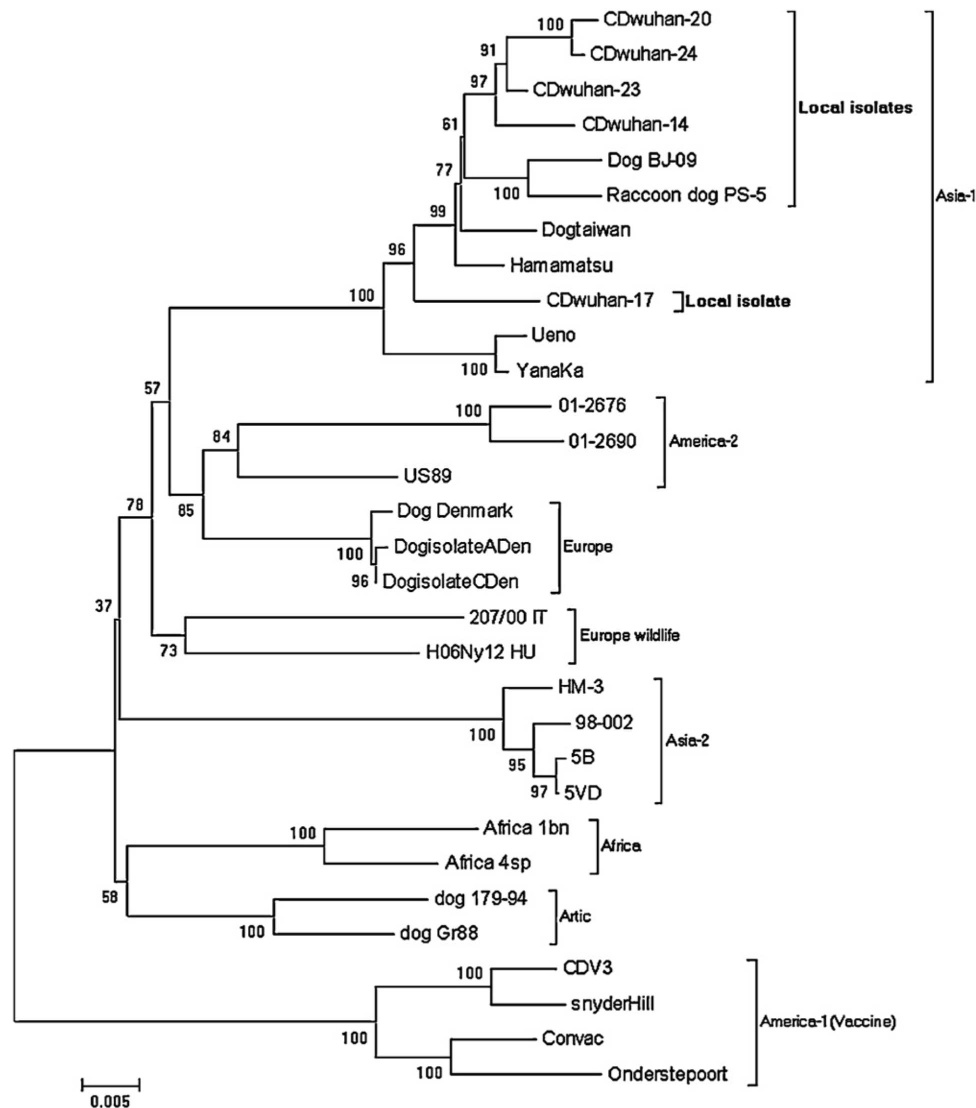


**Fig. 5.** The phylogenetic relationships among 27 CDVs based on the nucleotide sequence of H gene. Distance values were calculated by the Clustal W program within the MEGA 4.0 software package. The neighbor-joining algorithm was used to generate the tree. Bootstrap values were calculated on 1000 replicates. The local isolates indicate the 7 Chinese wild-type CDV strains analyzed in this study. GenBank accession numbers of the reference strains are as follow: CDV3 (DQ778941), Onderstepoort (AF378705), Convac (Z35493), SnyderHill (AF259552), Yanaka (D85755), Ueno (D85753), Hamamatsu (D85754), Dog98-002 (AB025270), Dog5B (AY297453), DogHM-3 (AB040767), Dog5VD (AY297454), DogTaiwan (AY378091), DogisolateADen (AF478543), DogisolateCDen (AF478547), Dog Denmark (Z47761), US89 (Z47764), Africa 1bn (FJ461713), Africa 4sp (FJ461715), dog GR88 (Z47760), dog 179-94 (DQ226087), 01-2676 (AY498692), 01-2690 (AY465925), H06Ny12HU (DQ889189)and 207/00 IT (DQ228166).

diagnoses between wild-type isolates and vaccines have been established. For example, an RT-PCR lineage-genotyping system based on specific nt polymorphisms scattered over the H gene has been developed. This system is used to characterize major CDV lineages, such as European, Asia-1, Asia-2, Arctic, and Vaccine strains (Martella et al., 2007). In another multiplex PCR assay, primers target the H gene to distinguish field strains from China and vaccine-like strains, such as Onderstepoort (Si et al., 2010). A multiplex amplification refractory mutation system PCR is also used to differentiate seven CDV field isolates in Taiwan (Asia-1 lineage) from vaccine strains based on the untranslated region region between the M and F genes (Chulakasian et al., 2010). Compared with these CDV differentiation assays, the presently proposed method may be conducted more easily and quickly. It making up by two separate steps RT-PCR assays to reduce contamination percentage and time-less in one PCR procedure but not multiplex or nested RT-PCR. CDV field isolates are also more easily distinguished from CDV commercial vaccines used in China. Unfortunately, the present method cannot detect mixed populations of CDV wild-type and vaccine strains, although these are rarely seen in animals. However, the method is still a suitable and easy diagnostic tool for detecting CDVs in animals with suspected CDV disease.

In the current study, phylogenetic analysis indicated the close relationship of CDV field strains with one another according to geographical source. The results confirmed and extended those previously reported in other global locations (An et al., 2008; Bolt et al., 1997; Haas et al., 1997; Iwatsuki et al., 1997; Pardo et al., 2005; Woma et al., 2010). All the CDV field strains analyzed in the present study could be grouped into a phylogenetic cluster clearly separate from the CDV vaccine strains, and they were characterized as having Asia-1 genotypes. This genotype appears to be predominant throughout China, and is infecting domestic dogs as well as raccoon dog species. Asia-1 CDV strains have also been detected in Japan (Mochizuki et al., 1999), Taiwan (Chan et al., 2009; Lee et al., 2010), and Korea (An et al., 2008).

In summary, the combined RT-PCR test developed in the current study is a suitable diagnostic tool for detecting and differentiating wild-type and vaccine strains of CDV.

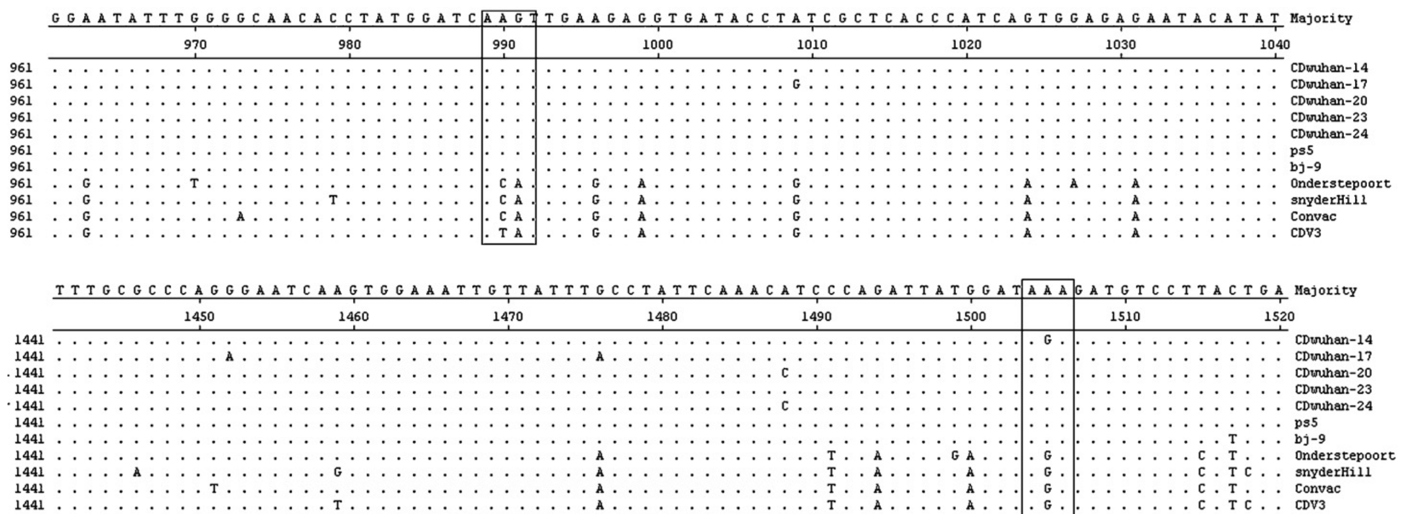

**Fig. 6.** Sequence alignment of partial hemagglutinin gene. The partial H gene nucleotide sequences of field strains from China and commercial CDV3 vaccine were analyzed. The numbering starts at the position nucleotide 961 and 1441 of the H gene. Only amino acids that differ from the majority sequence are shown. identical residues are represented by dots. The substitution of the AG and A present in the field strains, which was used to design the differentiating primers for RT-PCR, is indicated by a square box.

| Lineage | Strain | 990 | 1506 |
|---|---|---|---|
| Asia-1 | CDwuhan-17 | ……AGT…… | ……AA…… |
| | CDwuhan-24 | ……AGT…… | ……AA…… |
| | PS-5 | ……AGT…… | ……AA…… |
| | BJ-9 | ……AGT…… | ……AA…… |
| Asia-1 Japan isolate | YanaKa | ……AGT…… | ……AA…… |
| | Ueno | ……AGT…… | ……AA…… |
| Asia-2 | 5VD | ……AGT…… | ……AA…… |
| | 5B | ……AGT…… | ……AA…… |
| | HM-3 | ……AGT…… | ……AA…… |
| | 98-002 | ……AGT…… | ……AA…… |
| America-2 | 01-2676 | ……AGT…… | ……AA…… |
| | 01-2690 | ……AGT…… | ……AA…… |
| | US89 | ……AGT…… | ……AA…… |
| Europe | Dog Denmark | ……AGT…… | ……AA…… |
| | DogisolateADen | ……AGT…… | ……AA…… |
| | DogisolateCDen | ……AGT…… | ……AA…… |
| Europe wildlife | 207/00 IT | ……AGT…… | ……AA…… |
| | H06Ny12 HU | ……AGT…… | ……AA…… |
| Africa-isolate | Africa 1bn | ……GGT…… | ……AA…… |
| Vaccine | CDV3 | ……CAT…… | ……GA…… |
| | Onderstepoort | ……CAT…… | ……GA…… |
| | Snyder Hill | ……CAT…… | ……GA…… |

**Fig. 7.** Sequence comparison at nucleotide position 990nt and 1506nt of H gene with various CDV lineages. GenBank accession numbers of the reference strains are as follow, Asia-1 strains: CDwuhan17, CDwuhan24, PS-05, BJ-09 (GenBank: EU192013, EU191985, EU191988, EU327874, EU327875, EF596903, EF596904, EU934234), Asia Japan isolates: Yanaka (D85755), Ueno (D85753),Asia-2 strains: Dog5B (AY297453), DogHM-3 (AB040767), Dog5VD (AY297454), Europe strains: DogisolateADen (AF478543), DogisolateCDen (AF478547), Dog Denmark (Z47761), Europe wildlife strains: H06Ny12HU (DQ889189) and 207/00 IT (DQ228166), America-2 strains:US89 (Z47764), 01-2676 (AY498692), 01-2690 (AY465925), Africa isolates: Africa 1bn (FJ461713), and America-1 strains (contain vaccines): CDV3 (DQ778941), Onderstepoort (AF378705), SnyderHill (AF259552). Omitted sequences are represented by dots.

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