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Direct colorimetric detection of canine distemper virus by RT-PCR combined with gold nanoparticles

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Abstract:*Canine distemper is an acute systemic disease caused bycanine distemper virus (CDV), a RNA virus. CDV infection results in progress neurological signs of central nervous systemsystemically through the respiratory, digestive systems, and surrounding lymph tissues. It is therefore important to make an accurate rapid diagnosisin the early stages of the infection to prevent the disease from spreading. In this study, a rapid and simple gold nanoparticle (AuNP) colorimetric method was developed for CDV detection, named RT-PCR/AuNP assay. The assay is based on RT-PCR using specifically thiol-labeled primers to amplify the specific gene fragment of CDV. After mixing thiolated PCR amplicons with AuNP, and then the RT-PCR-AuNP products are formed by sulphur-gold linkage. The resulting RT-PCR-AuNP products are more salt-tolerant to induce aggregate of AuNP and the color of AuNP remains red with the PCR amplicons presence. Therefore, detection of CDV can be seen with naked eyes based on colorimetric testing and more accurate analysis can be achieved by spectrum measurement further. The limit of detection of RT-PCR/AuNP assaywas as low as 10^0 $TCID_{50}$/mL of CDV and the CDV could be easy detected at concentrations > 10^2 $TCID_{50}$/mL. For specificity test, the colorimetric method was also used to distinguish CDV from other canine pathogens. The clinical samples of CDV infection were successfully detected to demonstrate that the developed method is rapid, specific and potential for routine use in the clinical detection of CDV.*

Keywords:*Canine distemper virus; Colorimetric detection; Dog; Gold nanoparticle; RT-PCR*

I. Introduction

Dogs (*Canis lupus familiaris*) are widely recognized as the commonest petsas well as household companion animalsin human societies, and the issue of dogs healthy is taken seriously in the world.For many diseases in dogs, canine distemper is a highly contagious viraldiseaseof dogs caused by the canine distemper virus (CDV)[1].CDV infections can induce lethal systemic, respiratory, nervous and gastrointestinal manifestations to cause high morbidity and mortalityin unvaccinated dogs, puppies between the age of 3 and 6 months, and other carnivoresall over the world.

Canine distemper virus (CDV), a member ofthe *Morbillivirus* genus of the family *Paramyxoviridae*[2], was first isolated in 1905. The genome of CDV is approximately 15.7 kb in length and consists of a single-stranded, negative-sense RNA encoding for six structural proteins — nucleocapsidprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin (H) and large polymerase protein (L).The RNA genome is tightly associated with NP, L and P; however, the H and F are contained in envelope to involve in the virus infection and are antigenic determinants[3]. Nucleocapsid protein, the most abundant viral protein, can induce specific humoral and cellular immune response against CDV and measles virus (MV) in mice [4, 5] and the investigation of antigenic regions of NP had been located by Yoshida *et al.* [6].Therefore, the investigations of NP play an important role in CDV diagnosis and vaccine production [7, 8].

Some conventional methods have beenused to detect CDV includeenzyme-linked immunosorbent assay (ELISA)[9, 10]and nucleotide acids detections [11]. Many of these methods are effective and accurate in detecting viral infections in the laboratory.However, they are often laborious, time-consuming and expensive. With advances in molecular detection techniques, various RT-PCR methods have been established for CDV diagnosis with a varying degree of sensitivity and specificity[12, 13, 14]. Therefore, arapid, robust, sensitive and specificmethod isneeded for CDV detection from clinical specimens.In this study,the developed direct colorimetric assay is sensitive, specific and rapid detection of RT-PCR amplified DNA of CDV and represents a reliable diagnostic tool to aid routine laboratory identification of this pathogen.

II. Materials and Methods

2.1 Materials

All oligonucleotide primers were synthesized by MDBio Inc. (Taipei, Taiwan). All other chemicals such as tetrachloroauric acid ($HAuCl_4 \cdot 3H_2O$), sodium citrate ($C_6H_5Na_3O_7$), and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 CDV strain and RNA preparation

The culture supernatant of CDV containing viral particlesat $1 \times 10^6 TCID_{50}$/mL was kindly provided by Dr. C. K. Chuang of the Animal Technology Laboratories, Agricultural Technology Research Institute. The CDV suspension was seriallydiluted tenfold (10^5–$10^0 TCID_{50}$/mL), and the CDV RNA was prepared from 100 μL of CDV supernatant usingViral Nucleic Acid Extraction Kit II (GeneDirex, Las Vegas, NV, USA),according to the manufacturer's instructions. The extracted RNA was subjected toRT-PCR.

2.3Primers

The oligonucleotide primers used for amplification of the CDV nucleoprotein gene were as follows:
CDV-N-PI-F: 5'-ACA GGA TTG CTG AGG ACC TAT-3' (21-mer)
CDV-N-PI-R: 5'-CAA GAT AAC CAT GTA CGG TGC-3' (21-mer)
The sequences of the primer pair were designed according to the description of Frisk *et al*. [15] and the expected length of PCR amplicons was 287-bp. For the AuNP-based colorimetric assay, the primer pair was synthesized and labeled with thiol (-SH) at the 5'-end, respectively.

2.4RT-PCR

For reverse-transcription (RT), the reaction was performed by using MMLV First-Strand Synthesis Kit (GeneDirex). Briefly, 10 μL RNA was mixed with 2.5 mM Oligo $(dT)_{20}$ and 1.25 mM dNTP mixthat were denatured at 65°C for 10 min and immediately placed on ice. Then, the RT-Mix solution consisting of1× 1st strand synthesis buffer, 0.01 M of DTT, 2U RNase inhibitor and 200 U of MMLV Reverse Transcriptase to a final volume of 20 μL. After mixing well, the mixture was incubated at 37°C for 1h and then at 70°C for 10 min to stop the reaction.

Polymerase chain reaction (PCR)was performed in a 25-μLvolumecontaining 1μL of cDNA from RT; 4 μM of each primer; 200 μM each of dATP, dCTP, dGTP, anddTTP (Promega, Madison, WI, USA);5 μL of 5× PCR buffer (100 mMTris-HCl, 9 mM $MgCl_2$, 110 mM NH_4Cl, 110 mMKCl, 0.3% IGEPAL CA-630, and 0.25% Tween20, pH8.9);0.5 U of *OneTaq* DNA polymerase (New England BioLabs, Ipswich, MA, USA); and H_2O. The PCR conditionswereasfollows: denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 sec, annealing at 59°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for a further 10 min. The amplified PCR products were purified with PCR Clean-Up& Gel Extraction Kit (GeneDirex) and analyzed by electrophoresis on a 2% agarose gel containing 0.5 μg/mL ethidium bromide

2.5Preparation of AuNP

The 20-nm AuNP were prepared by means of sodium citrate reduction method described by Chen *et al*. [16]. 50 mL of 1 mMtetrachloroauric acid solution was heated and stirred vigorously until the solution was boiling. Then, 5 mL of 38.8 mM sodium citrate was added as quickly as possible into the boiled chloroauric acid solution. Finally the tetrachloroauric acid solution turned into claret and left to cool at room temperature. The absorption spectra of the AuNP were measured by a spectrophotometer (SpectraMax 190; Molecular Devices Corp., Sunnyvale, CA, USA).

2.6 Colorimetric assay with AuNP

For the colorimetric assay, 10 μL of the RT-PCR product was mixed with 20 μL of the gold colloid. After incubating at room temperature for less than 1 min, 2 μL of 5 M NaCl was added and observed the mixture's color change without any visual aid. Furthermore, the mixed solution was also quantified by spectrophotometer. The difference of AuNP aggregation degree was between absorbance at wavelength 520 (A_{520}) and 620 (A_{620}), the ratio of A_{520}/A_{620} was selected to evaluate the performance of developed RT-PCR/AuNPassay[16, 17].

III. Results and Discussion

3.1 Establishment of the RT-PCR/AuNP assay for CDVdetection

A schematic presentation of the RT-PCR assay for CDV with the specific thiol-labelled primers for amplifying CDV DNA fragments and reacting with AuNPwas illustrated in Fig. 1. In this RT-PCR/AuNP assay, the viral RNA of CDV was first extracted from the samples. The viral cDNA was obtained by RT and used to get the specific and thiolated287-bp double-strand DNA (dsDNA) fragment by PCR.The RT-PCR amplicons

were then reacted with AuNP. AuNP can cap onto the 5' thiol-labelled dsDNA via sulphur-gold linkage, and stabilize the AuNPin the solution with 0.5 N NaCl to avoid AuNP aggregation. That is the RT-PCR-AuNP products are more salt-tolerant to induce aggregate of AuNP. The stabilizing AuNP in solution would retain the initial red color (i.e., maintaining AuNP original plasmon resonance at A_{520} nm). When AuNP do not cap onto dsDNA, such as no dsDNA or the dsDNA without thiol-label, the AuNP would aggregate and the aggregation of AuNP is corroborated by visible spectra, where an intense plasmon resonance band appears at 600–650 nm (purplein color observed by naked eye) (Fig. 2).

Several detection methods have been developed to detect CDV antibodies, proteins and nucleic acids, and many of these tests are effective and accurate in detecting the viral infection in laboratory [9, 10, 11]. However, they require expensive equipment and are often laborious and time-consuming. Early and rapid diagnosis is necessary, so that CDV-infected dogs can be isolated to prevent the spread of the disease and to administer supportive treatment for reducing morbidity and mortality.

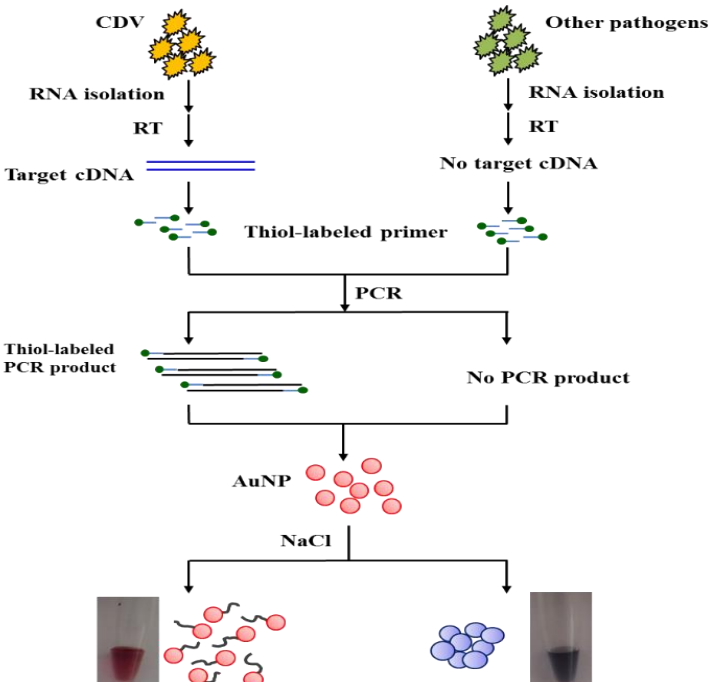

Figure 1: Scheme of direct colorimetric detection of canine distemper virus (CDV) by RT-PCR combined with gold nanoparticles (AuNP). It is defined as RT-PCR/AuNP assay for CDV detection.

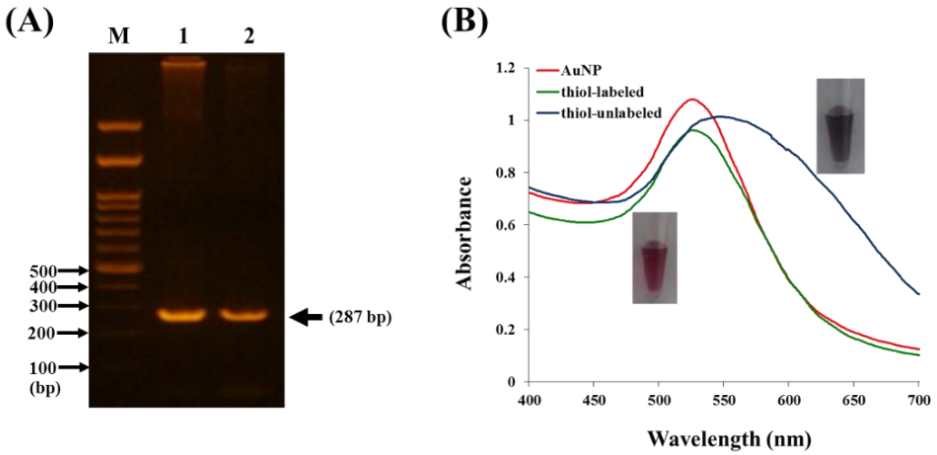

Figure 2: The specifically amplified CDV DNA fragments obtained by RT-PCR with the thiol-labeled primers (lane 1) and with thiol-unlabeled primer (lane 2) (**A**). Absorbance curves (A_{400}-A_{700}) ofAuNP only, CDV DNA fragments with the thiol-labeled primer reacted with AuNP (optical color of the solution is red), and CDV DNA fragments with the thiol-unlabeled primer reacted with AuNP (optical color of the solution is purple).

3.2. Sensitivity of RT-PCR/AuNP assay for CDV detection

To determine the sensitivity of the CDV detection by RT-PCR/AuNP assay, the CDV stock (10^6TCID$_{50}$/mL) was seriallydiluted tenfold and the CDV RNA was prepared from 100µL of CDV supernatant.Each viralRNA (10µL) was used as a template for RT-PCR. Anamplified 287-bp fragment from the CDVwas visible on agarose gel(Fig. 3A).The quantification resultsshowed that the RT-PCR/AuNPassay could easy detect CDVat concentrations>10^2TCID$_{50}$/mL(Fig. 3B and 3C), and the detection is linearly dependent on the concentrations between 10^2 – 10^4 TCID$_{50}$/mL of CDV (Fig. 3C). Additionally, the limit of detection of RT-PCR/AuNP assaywas as low as 10^0 TCID$_{50}$/mL of CDV(Fig. 3C).

In the present study, the CPV detection limit by RT-PCR/AuNP assay was 10^0TCID$_{50}$/mL. The result indicated that the sensitivity of RT-PCR/AuNP assay for CDV detection is similar to that of conventional molecular methods [18, 19]. However, the detected results of developed RT-PCR/AuNP assay could be easily visualized with naked eye.

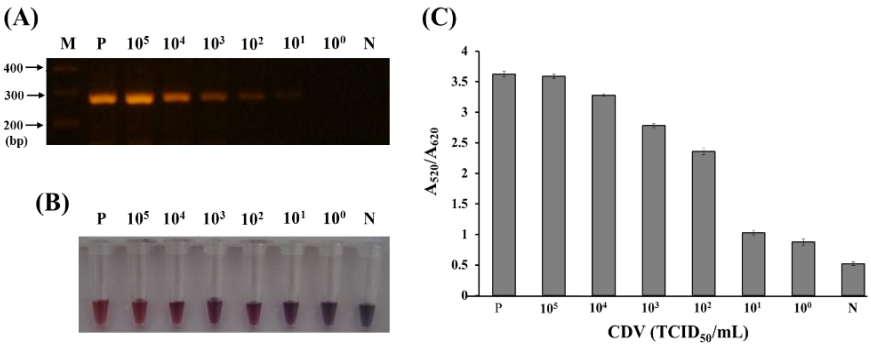

Figure 3: The specifically amplified CDV DNA fragments obtained from the CDVin the titer of 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 TCID$_{50}$/mL by RT-PCR with thiol-labeled primers (**A**). The positive (P) and negative (N) reactions were also performed. The amplified CDV DNA fragments reacted with AuNPwere optically observed (**B**). Absorbance curves were detected and the ratios of A_{520}/A_{620} of each determinant were calculated and compared (**C**).

3.3Specificity of RT-PCR/AuNP assay for CDV detection

In order to evaluate the specificity of established RT-PCR/AuNPassay, potential cross-reactionswere performed using DNA/RNA extracted from different pathogens, including CDV, canine parvovirus (CPV), infectious canine hepatitis virus (ICHV), *Bordetella bronchiseptica*, and*Leptospiracanicola*. Thiol-labeled RT-PCRamplicons were analyzed by 2% agarose gel electrophoresis and RT-PCR amplicons were reacted with AuNP.As shown in Fig. 4A, cross-amplification tests using templates from CPV, ICHV, *L.canicola*, and *B. bronchisepticas*howed that no PCR amplicons were detected, whereas the reaction using the CDV template showed a positive result. The same results were also observed in the RT-PCR/AuNP reacted solution; the positive test appeared only for CDV detectionjudged by naked eye(Fig. 4B; the color of the reaction solution is red) or by spectrophotometry (Fig. 4C; a significant high ratio of A_{520}/A_{620}). These results indicated that the RT-PCR/AuNPassaydeveloped in this study was specific for CDV detection.

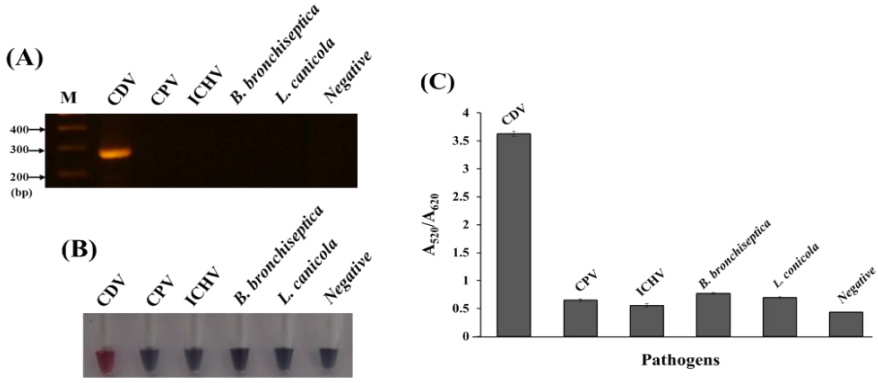

Figure 4: Specificity of the direct colorimetric detection of CDV by RT-PCR combined with AuNP (RT-PCR/AuNP assay) was performed. The specifically amplified CDV DNA fragments were obtained from the sample of CDV, but not from the samples of canine parvovirus (CPV), canine hepatitis virus (ICHV), *B. bronchiseptica* and *L. canicola* genomic DNA. The experimental evidence came from the sampleswere identified by the detections of agarose gel electrophoresis (**A**) and direct colorimetric observation (**B**), and spectrophotometric ratio of A_{520}/A_{620} (**C**).

3.4CDV detection in the dog fecal samples containing CDV

There were 9 dogs that were clinical diagnosed with CDV infection and the infected CDV were identified. The fecal samples from these9 dogs were sampled for the CDV detection by our established RT-PCR/AuNPassay. The detective results showed that all of the detections are positive reaction. The specific CDV PCRamplicons were present on 2% agarose gel (Fig. 5A) and the positive detections also could be easily read with naked eye (Fig. 5B).In the present study, the detection of CDV can be seen with naked eyes based on colorimetric testing and more accurate analysis can be achieved by spectrum measurement further.Currently, numerous methods utilizing AuNPs for detection are available as discussed and reviewed by several researchers [17, 20, 21]. The methods of AuNP-based optical biosensing detection still continues to be a promising tool for rapid and specific clinical and veterinary pathogen detection.

Molecular detection methods for CDV in animal fecal samples [22, 23]have been applied for laboratory diagnosis. However, these assays are time-consuming and require specialized equipment. Therefore, a novel and direct colorimetric detection of CDV by RT-PCR combined with AuNP method, which is applied to detect CDV in animal fecal samples within hour, is a specific, simple, and rapid alternative for CDV pre-screening test in the field. In the present report, we described the development of RT-PCR/AuNP diagnostic systems with an assay time of <3 h for CDV levels of clinical concern. Our results confirmed the results of recent reports that indicated advanced RT-PCR assays to be highly-sensitive methods[13, 14] that can be easily applied for the visual detection of clinical pathogens. Our results indicated that the RT-PCR/AuNPassay developed in the present study is applicable to CDV detection in naturally contaminated fecal samples and the assays could be completed within 3 h.

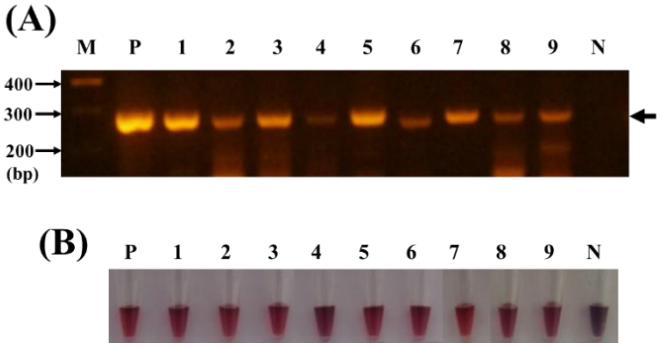

Figure 5: The fecal samples from the dogs that were clinical diagnosed with CDV infection were used for the detection by the direct colorimetric detection of CDV by RT-PCR combined with AuNP (RT-PCR/AuNP assay). The detective results showed that all of the detections are positive reaction.

IV. Conclusions

In conclusion, when RT-PCRwas combined with AuNP, the detection signal of PCR ampliconscould bespectrophotometrically obtained and easily read with the naked eye. The assays could be completed within 3 hr. Theresults indicated that a simple and cost-effective RT-PCR/AuNP-based technique can be developed into a rapid and reliable molecular diagnostic methodwith potential for routine use in the clinical detection of CDV and other veterinary clinical pathogens.

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