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Detection of canine distemper virus (CDV) through one step RT-PCR combined with nested PCR

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A one step reverse transcription PCR (RT-PCR) combined nested PCR was set up to increase efficiency in the diagnosis of canine distemper virus (CDV) infection after developement of nested PCR. Two PCR primer sets were designed based on the sequence of nucleocapsid gene of CDV Onderstepoort strain. One-step RT-PCR with the outer primer pair was revealed to detect 10^2 PFU/m*l*. The sensitivity was increased hundredfold using the one-step RT-PCR combined with the nested PCR. Specificity of the PCR was also confirmed using other related canine virus and peripheral blood mononuclear cells (PBMC) and body secretes of healthy dogs. Of the 51 blood samples from dogs clinically suspected of CD, 45 samples were revealed as positive by one-step RT-PCR combined with nested PCR. However, only 15 samples were identified as positive with a single one step RT-PCR. Therefore approximately 60% increase in the efficiency of the diagnosis was observed by the combined method. These results suggested that one step RT-PCR combined with nested PCR could be a sensitive, specific, and practical method for diagnosis of CDV infection.

Key words: CDV, RT-PCR, nested PCR

Introduction

Canine distemper (CD) is a worldwide, highly contagious disease in young dogs, particularly in 3 to 6 months of age, with high morbidity and mortality. It is manifested by a diphasic fever curve and acute rhinitis, and later by bronchitis, catarrhal pneumonia, severe gastroenteritis, and nervous signs [17]. The disease spread mainly in the winter since canine distemper virus (CDV), a member of the genus *Morbillivirus* of family *Paramyxoviridae*, could survive for a longer period of time under cold condition [11]. It is comparatively rare in many developed countries, being well-controlled through vaccination using the attenuated live virus [6]. However, in areas with unvaccinated populations, CD occurs whereever dogs are raised. Recently, many cases of CD have been reported regardless of the seasons in Korea.

Diagnosis of CD in acute or subacute form had been done usually based on clinical signs and history in unvaccinated puppies. But it was difficult to differentiate CD from other diseases such as kennel cough in the early stage. Serologic diagnosis might be accomplished through detection of anti-CDV IgM antibody [4, 9], but it still pose as a problem in vaccinated dogs due to a measurable IgM antibody titer to CDV within 3 weeks after vaccination [9]. Definitive diagnosis could be made through isolation of the virus or detection of CDV in epithelial cells after fluorescent antibody (FA) staining [6]. However, the virus isolation takes several days to weeks and is frequently not effective in acute stage of the infection [1, 15]. FA test was successful only during the first few days of acute signs of distemper [3, 7]. After a technique of *in vitro* DNA amplification with a thermostable DNA polymerase was introduced [13], it has been widely applied to diagnosis of several types of diseases including viral infection. Recently, infections of CDV [14] and other morbilliviruses [8, 10, 16] were also determined through reverse transcription PCR (RT-PCR). But detection of CDV with the RT-PCR was not satisfactory during the first and end stages of the infection. Therefore, in this study, to increase efficiency in the diagnosis of CD, a one-step RT-PCR combined with nested PCR was developed after the establishment of nested PCR.

Materials and Methods

Dogs

Five healthy, vaccinated dogs with an attenuated live canine distemper vaccine (DaeSung Microbiology co., Korea) and 61 affected dogs clinically suspected of CD

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were used. The clinically suspected dogs, prepared from Veterinary Medical Teaching Hospital of Seoul National University and local veterinary hospitals in Seoul area, Korea, revealed typical clinical signs of CD such as conjunctivitis, bronchitis, catarrhal pneumonia, gastroenteritis, and neurological disturbances.

Preparation of samples

Blood, ocular discharge, nasal discharge, saliva, and feces were collected from the vaccined dogs at 0, 2, 7, 14 days after vaccination. Blood samples from healthy and suspected dogs were also collected. Peripheral blood mononuclear cells (PBMC) were isolated from 2 m*l* of whole blood treated with anticoagulant (CPD-A1®, Green Cross co., Korea) by centrifugation over Ficoll-sodium diatrizoate solution.[c] Ocular and nasal discharges, saliva, and feces were swabbed and then eluted with 0.5 m*l* phosphated buffered saline (PBS). Supernatants of urine collected through cystocentesis were obtained by centrifugation for 10 min at 12,000 rpm.

Other common canine viruses (parainfluenzavirus 2, canine coronavirus, infectious canine hepatitis virus, and canine parvovirus) were also prepared (DaeSung Microbiology co., Korea). To determine the sensitivity of one-step RT-PCR and nested PCR, CDV vaccine strain was diluted tenfold from 10^3 to 10^0 PFU/m*l* and used for isolation of RNA.

Isolation of RNA

Total RNA was prepared using acids guanidium thiocyanate-phenol-chloroform extraction method[d] following the manufacture's instruction (Total RNA Isolation Reagent, Advanced Biotechnologies Ltd., Epsom, UK) and then washed with 75% ethanol.

Amplification of CDV Nucleocapsid (NP) gene

Primers specific to nucleocapsid gene of CDV Onderstepoort strain were designed (Fig. 1)[14] and synthesized with a DNA synthsizer. The synthesis of first strand cDNA was carried out in a 20 μl reaction mixture containing 13.3 μl of the annealed RNA-primer mixture (50 pmol outer primer set and 12.3 μl RNA extract), 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$, 1 mM dNTP mix, 1 U/μl RNase inhibitor (Takara, Japan), and 0.32 U/μl AMV (avian myeloblastosis virus) reverse transcriptase (Takara, Japan). cDNA was synthesized through incubation at 42°C for 50 min, and the enzyme was denatured by heating at 69°C for 5 min. The cDNA was used as a template in the 50 μl PCR reaction with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 50 pmol 1st forward and reverse primers, and 0.05 U/μl *Taq* polymerase (Takara, Japan). PCR amplification of CDV was carried out in 30 sequential cycles at 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min.

One-step RT-PCR was performed in the 50 μl reaction volume with 20 μl RNA extract, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTP mix, 50 pmol first forward and reverse primers, 0.13 U/μl AMV reverse transcriptase, 0.8 U/μl RNase inhibitor, and 0.05 U/μl *Taq* polymerase. One-step RT-PCR amplification of CDV was carried out in 30 sequential cycles at 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min after incubation at 42°C for 50 min for the synthesis of cDNA.

In nested PCR, 1 μl (the detected case in first RT-PCR)

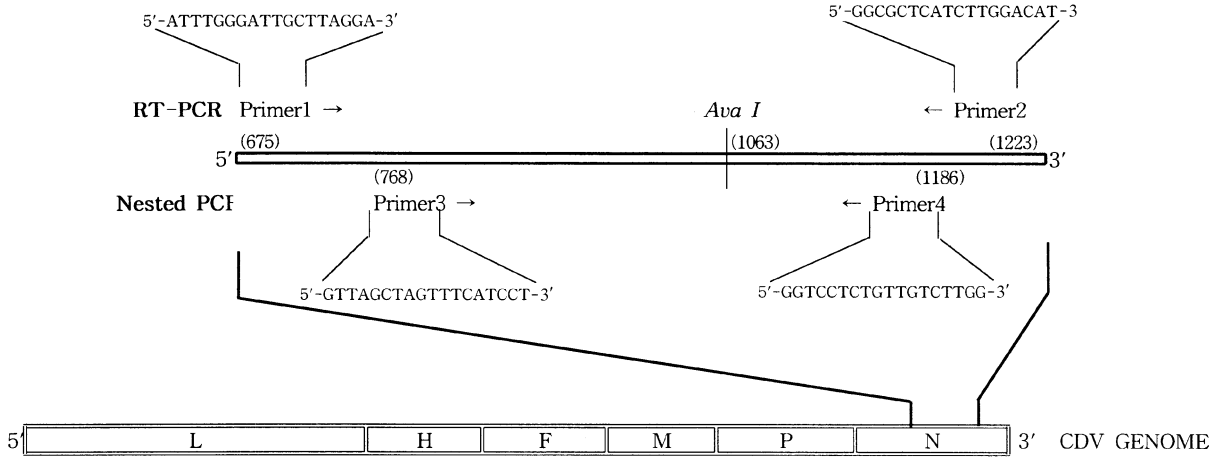

Fig. 1. Diagramatic representation of CDV NP gene to be amplified by RT-PCR and nested PCR and position of the outer and inner primer pairs. Primers were designed from CDV nucleocapsid gene of Onderstepoort strain. The 549 and 419 bps fragments of nucleocapsid gene were amplified by RT-PCR and nested PCR with the outer (primers 1 and 2) and inner primer pairs (primers 3 and 4), respectively. Primer 1 = outer forward primer positioned at 675 to 692, primer 2 = outer reverse primer positioned at 1206 to 1223, primer 3 = inner forwared primer positioned at 768 to 785, primer 4 = inner reverse primer positioned at 1169 to 1186. Amplified products were identified by restrionion endonuclease, Ava I (restriction site: 1063). L = large virus-specific RNA-directed RNA polymerase protein, H = hemagglutinin protein, F = fusion protein, M = matrix protein, P = phosphoprotein, N = nucleocapsid.

or 5 μl (non-detected case) of the PCR products amplified with the outer primer pairs was used as a template and amplified with the inner primer pairs using the same procedure described in the first PCR after cDNA synthesis. Amplified PCR products were visualized under UV illuminator after Etbr staining.

Analysis of PCR product

The PCR products were analyzed by 1.5% agarose gel electrophoresis after digestion with restriction endonuclease, *AvaI*.[e] The DNA fragment of the first PCR was cloned into plasmid using a TA-cloning method (TOPO™ TA Cloning®, Invitrogen, USA).[f] Extracting plasmid DNAs (GENOMED plasmid kit®, Genomed, Germany), both strands of plasmid inserts were sequenced using the dideoxy chain termination method (Dye Terminator AmpliTaq kit®, PE Applied Biosystems, USA). The similarity of nucleotide sequence of PCR product obtained through sequencing analyzer was calculated (BLAST program, http://www.ncbi.nlm.nih.gov/BLAST).

Serologic test

Serum neutralization test (SN) was performed using the Chalmers and Baxendale's method[6] with a minor modification. Sera were collected from five vaccinated dogs weekly for three weeks after vaccination. Heat inactivated and serially diluted sera were mixed with equal volume of CDV suspensions containing 200 $TCID_{50}$/m*l*. After incubation for 1 h at 37°C, 0.1 m*l* of the mixtures were inoculated on to monolayered Vero cell and incubated at 37°C for four days in 5% CO_2. SN titer was determined by calculating the 100% inhibition dilution dose of cytopathic effect.

Results

Amplification of CDV NP gene by a RT-PCR and nested PCR

Five-hundred and forty-nine bp fragment of NP gene was successfully amplified from tissue culture fluid containing CDV vaccine strain (Lederle; 10^3 PFU/m*l*) by a RT-PCR with the outer primer pair (Fig. 2). From 10 cases of clinically suspected dogs for CD, only 8 dogs were found positive by a previous RT-PCR. However, the gene was not detected from 2 cases of the positive dogs after 9 days with the same RT-PCR.

With inner primer pair for nested PCR to increase the sensitivity and specificity, 419 bp fragment was successfully amplified from 1 μl of the first PCR product (Fig. 2). With the nested PCR, the positive band of 419 bp was successfully amplified from all samples clinically suspected of CD including 2 negative products in the reexamination of the positives (data not shown).

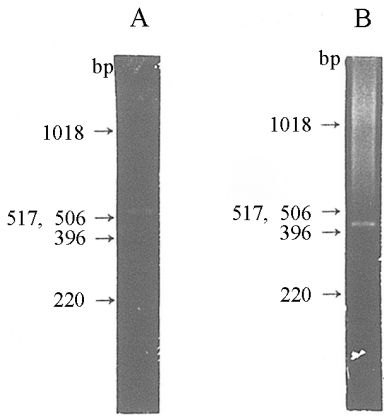

Fig. 2. Amplification of CDV NP gene by previous RT-PCR and nested PCR. Five-hundred and forty-nine bp (panel A) and 419 bp (panel B) fragments were successfully amplified from tissue culture fluid containing CDV vaccine strain (Lederle; 10^3 PFU/ml) and visualized by ethidium bromide staining.

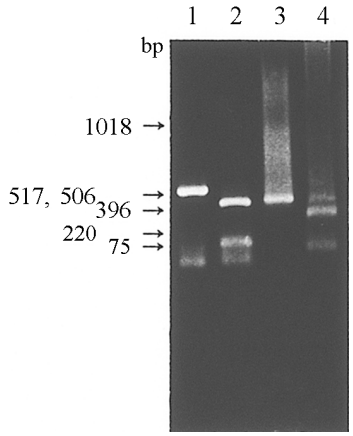

Fig. 3. Detection of CDV NP gene by one-step RT-PCR combined with nested PCR. One-step RT-PCR and nested PCR products were visualized by ethidium bromide staining and treated with restriction endonuclease, Ava I. Lane 1: one-step RT-PCR product (549 bp), lane 2: digestion product of one-step RT-PCR product (389 & 160 bp), lane 3: nested PCR product (419 bp), and lane 4: digestion product of nested PCR product (296 & 123 bp band).

Amplification of CDV NP gene by one-step RT-PCR with nested PCR

Five-hundred and forty-nine and 419 bp fragments of NP gene were successfully amplified by one-step RT-PCR and nested PCR with the outer and inner primer pairs, respectively (Fig. 3). In the digestion of the PCR products with *Ava I*, the products with outer and inner primer sets were 389 and 160 bp fragments and 296 and 123 bp fragments, respectively (Fig. 3). The nucleotide sequence of the one-step RT-PCR product showed a 98% identity with the sequence of CDV NP gene from a previous report.[14] In the sensitivity of one-step RT-PCR and nested PCR, the

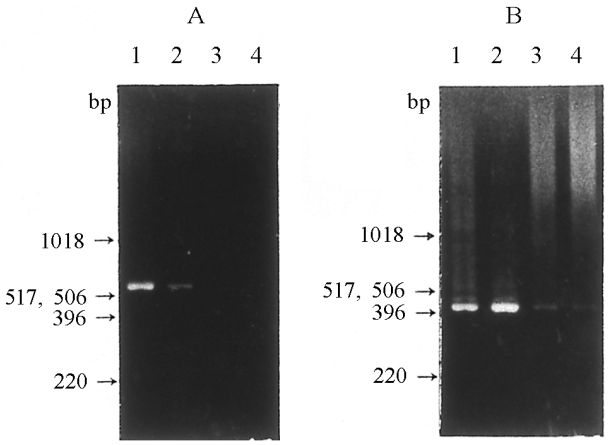

Fig. 4. Sensitivity of one-step RT-PCR and nested PCR. Lanes 1-4 of panel A: amplified products with one-step RT-PCR using virus titier 10^3 to 10^0 PFU/ml and lanes 1-4 of panel B: amplified products with nested PCR using PCR products of one-step RT-PCR for virus titer 10^3 to 10^0 PFU/ml.

detection limits were 10^2 and 10^0, respectively (Fig. 4). PBMC and normal body secretes (ocular discharge, nasal discharge, saliva, feces, and urine) of healthy dogs and other common canine viruses (parainfluenzavirus 2, canine coronavirus, infectious canine hepatitis virus, and canine parvovirus) were also tested with the same primers. No detectable bands were produced by one-step RT-PCR and nested PCR (data not shown).

Detection of CDV in vaccinated dogs

CDV NP gene from 5 vaccinated dogs with one-step RT-PCR and nested PCR was detected at 2 days after vaccination, but not at 7 days, in PBMC only by one-step RT-PCR. However, combined with nested PCR, 4 of the 5 samples were positive at 7 days. It was also detected with combined nested PCR at 2 and 7 days in other samples (ocular discharge, nasal discharge, saliva, feces, and urine) with various ratios. However, no amplified band was observed after 14 days (Table 1). SN titer was >250 at 1-2 weeks and 128 at 3 weeks after vaccination in all vaccinated dogs.

Detection of CDV in clinically affected dogs

Of the 51 PBMC samples from dogs with the typical clinical signs of CD, the amplified NP gene was detected in 45 dogs by one-step RT-PCR combined with nested PCR. Of the 45 positive samples, however, only 15 samples were revealed as positive through single one-step RT-PCR. The last 6 cases shown local myoclonus of temporal muscle or thoracic and pelvic limb were also suspected of being infected with CDV even though the gene was not detected by one-step RT-PCR combined with nested PCR.

Discussion

CD is the most important viral, contagious disease found in dogs, particularly 3 to 6 months of age, with high morbidity and mortality. Diagnosis of CD in acute or subacute forms had been done usually based on clinical signs such as conjunctivitis, bronchitis, catarrhal pneumonia, gastroenteritis, and neurological disturbances. However, some problems arose in the differentiation with other diseases such as kennel cough or other clinical forms such as delayed-onset and chronic distemper encephalitis, among others. Although detection of anti-CDV IgM antibody, FA, and virus isolation had been used, these methods also had several problems such as time-consuming, time-limitation, and cross-reaction in vaccinated dogs in the diagnosis of CD. Therefore, development of a sensitive, specific, and practical method was required. With growing knowledge in molecular biology, a RT-PCR was developed to detect CDV. However, this method still had problems in sensitivity and specificity due to contamination error since the reaction was carried out in separate tubes for RT and PCR. We established a one-step RT-PCR. Moreover, a nested PCR was developed from the product of one-step RT-PCR. Sensitivity of the one-step RT-PCR combined with nested PCR increased hundredfold than the previous PCR using culture supernatant containing CDV vaccine strain. The

Table 1. Detection of canine distemper virus in vaccinated dogs by one step RT-PCR combined with nested PCR

Samples	Days after vaccination							
	0		2		7		14	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
PBMC	0/5*	0/5	5/5	5/5	0/5	4/5	0/5	0/5
Conjunctival swab	0/4	0/4	0/4	4/4	0/4	2/4	0/4	0/4
Nasal discharge	0/4	0/4	0/4	4/4	0/4	1/4	0/4	0/4
Saliva	0/4	0/4	0/4	3/4	0/4	2/4	0/4	0/4
Feces	0/4	0/4	0/4	3/4	0/4	2/4	0/4	0/4
Urine	0/4	0/4	0/4	2/4	0/4	0/4	0/4	0/4

*No. of positive/No. of tested;
1st = one-step RT-PCR, 2nd = nested PCR.

sensitivity was confirmed using blood samples of dogs clinically suspected of CDV infection. Specificity of the PCR was confirmed by PBMC and body secretes of healthy dogs and other viruses which could infect dogs and showed similar clinical signs with CD (parainfluenzavirus 2, canine coronavirus, infectious canine hepatitis virus, and canine parvovirus). Identity of the PCR products was confirmed by digestion with *AvaI* and nucleotide sequencing of the PCR products.

Within 6 days after infection, all lymphatic tissues are infected, and viremia is developed. Dogs without antibody against CDV die approximately 3 weeks after exposure, showing widespread distribution of virus in lymphatic tissue, epithelium, and brain, with signs of illness. But viral antigens disappear within 2 weeks if infected dogs obtain high serum antibody titer.[2] These phenomena were confirmed in this experiment through detection of CDV from PBMC and body secretes of vaccinated dogs. These results from vaccinated dogs suggested the importance of determining the time period for the effective application of the PCR method. In the comparision of the diagnostic efficency of the two PCR methods with 51 PBMC samples suspected of CD, the efficiency of one-step RT-PCR combined with nested PCR was increased up to 60%. Although the one-step PCR combined with nested PCR was found to be the most sensitive method to detect CDV from the specimen, further studies to find the proper time to take samples from dogs should be performed.

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