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Strain differentiation of canine distemper virus by reverse transcriptase polymerase chain reaction and restriction fragment length polymorphism analysis

Dong-jun An, Jae-young Song, Joung-bok Lee*, Jong-hyeon Park,
Jin-ho Shin, Yong-hwan Kim**, Soo-hwan An

National Veterinary Research and Quarantine Service, Anyang, Korea
Laboratory of Microbiology & Infectious Disease,
*School of Veterinary Medicine, Konkuk Universty, Seoul**
*College of Veterinary Medicine, Seoul National Universty, Suwon, Korea***
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Abstract : To detect CDV RNA in clinical samples and differentiate prevailing CDV virulent strains affecting susceptible animals from attenuated vaccine strains, we performed RT-PCR, RFLP, and sequencing. CDV specific primers were generated from the middle part of nucleocapsid gene. The expected size of PCR products, 519 bp, was observed in tissues of Jindo dog, poodle dog, badger, fourteen of nineteen blood samples as well as 5 vaccine strains including domestic and imported products. The PCR products obtained from tissues and PBMCs of infected animals were digested to 317- and 202-bp fragments by *Bam* HI, but the products obtained from four of five vaccine strains and Lederle strain were not digesed by *Bam* HI. Only one vaccine strain of which the PCR products were digested by *Bam* HI was confirmed as imported vaccine, modified Synider Hill strain. Based on seqencing data obtained from the 519-bp products, it was confirmed that *Bam* HI restriction site tends to be conserved in field isolates compared to the commercially available attenuated vaccine strains. Partial nucleotide sequences of CDV NP gene obtained from tissues of Jindo dog, poodle and badger shared 100% homology each other, whereas the nucleotide sequences showed 96.3, 96.5, 93.6 and 93.4% homology with Yanaka (virulent), Han95 (virulent), Lederle (attenuated) and Onderstepoort (attenuated) strain, respectively.

Key words : CDV NP, RT-PCR, RFLP.

Address reprint requests to Dr. Dong-jun An, National Veterinary Research and Quarantine Service, Anyang 6-Dong, Anyang 430-016, Republic of Korea.

Introduction

Canine distemper (CD), caused by canine distemper virus (CDV), is an acute to subacute contagious systemic disease in dogs, fox, raccoon, mink and ferret with a high mortality rate and occurs throughout the world[1,2]. Canine distemper has been well controlled in the developed countries with live attenuated vaccines[3]. However, a number of recent outbreaks of CD have been observed worldwide[4~8]. Epidemiological research on CD in Japan as well as in European countries revealed that the incidence of CD increased in dogs which were vaccinated as well as unvaccinated[6~8]. An analysis in Korea revealed that prevalence of CDV antibody increased up to 80.9% in 1995 whereas there was 49.0% in 1991. Kim *et al* reported that a wild raccoon was infected with CDV[9~11]. A number of recent outbreaks of CD was reported in Japan. These data suggest that the new canine distemper viruses may emerge regardless to vaccination[8,9,12]. It is supported by the evidence that the haemagglutinin of an isolate consisted of potential N-linked glycosylation[13].

The currently available live-attenuated vaccine efficiently protects dogs, but incidence of canine distemper has been reported in cities. These outbreaks may be either due to maternal antibody, or to newly emerging CDV strain. This study was aimed to detect canine distemper virus by RT-PCR and differentiate between vaccine and wild type of CDV strains.

Materials and Methods

Viruses and clinical samples : Jindo- and poodle dogs hospitalized with neurologic symptom in an animal clinic in Seoul transported to the National Veterinary Research and Quarantine Service (NVRQS), Anyang. Those dogs were euthanized and clinical lesions were collected. A badger was directly tranported to the NVRQS for precise diagnosis and also its lesion was collected. Nineteen dogs were suspected with CDV infection in hospital of Seoul National University and animal clinics in Seoul. Peripheral blood mononuclear cells (PBMCs) were obtained from the patient dogs. For the reference, 3 different lots of commercially available domestic vaccines and 2 different lots of imported vaccines were purchased. The Lederle strain (ATCC VR-128) was obtained from American Type Culture Collection. RT-PCR was performed using tissues, PBMCs, vaccines and Lederle strain.

Synthesis of oilgonucleotide primer : Primers were generated based on the nucleocapsid gene of CDV. Forward primer was located at nucleotide (nt) 662 with the sequence of 5'-CCG GAT CCA CAG GAT TGC TGA GGA CCT A-3'(CDNPF) and reverse primer at nt 1181 with that of 5'-CCG GAT CCA CTA GCT GAG CTT CCT CCT T-3' (CDNPR) (Table 1).

RT-PCR : The clinical samples were emlusified and supernatants were collected for synthesis of first strand cDNA. Blood was collected into heparinized tubes and sedimented by centirfuging at 1,200× g for 10 min. And then buffy coat layer was collected and loaded onto 250μl histopaque-1077, histopaque-1119 (Sigma). PBMCs were separated by spinning at 1,500× g for 30 min. The PBMCs was washed twice with PBS and total RNA was extracted using Ultraspec™-Ⅱ RNA Kit (Biotecx Laboratories, INC.). Briefly, three hundred microliters of sample suspension was mixed with 1 ml of Ultraspec™-Ⅱ regent, and added with 100μl of chloroform, and then vortexed thoroughly. Tubes were kept on ice for 5 min, and centrifuged at 12,700× g for 15 min. About 300μl of aqueous phase was carefully transferred into a fresh tube, and 150μl of isopropanol and 20μl of RNA Tack™ Resin were added and vortexed for 30 s.

Table 1. Oligonucleotide primers for amplification of nucleocapsid gene

CDV gene	Primer sequence 5'→3'	Location (nt)	Length o fragment (bp)
Nucleocapsid	CCGGATCCACAGGATTGCTGAGGACCTA	662	519
	CCGGATCCACTAGCTGAGCTTCCTCCTT	1181	

The tube was spun at 12,700× g for 1 min and its pellet was washed twice with 1 ml of 75% ethanol. The pellet was dried and resuspended in 20μl of diethyl pyrocarbonate (DEPC)-treated water (Biotecx, BL-5612). To synthesize first strand cDNA, the pelleted total RNA was mixed with 1 μl of CDNPF (100 pmole/μl) and CDNPR primer (100 pmole/μl) and boiled for 5 min. Ten microliters of 5× first strand buffer, 5μl of 0.1M DTT, 2μl of 10mM dNTP, 1 μl of RNasin (40U/μl) and 1 μl of M-MLV reverse transcriptase (200U/μl, BRL) were added and incubated at 37℃ for 1 h. The 10μl of first strand cDNA was transferred into a new tube containing 64μl of H_2O, 10μl of 10× PCR buffer (200mM Tris-HCl, 500mM KCl), 12μl of 8mM $MgCl_2$, 2 μl of 10mM dNTPs, 1 μl of CDNPF (100 pmole/μl), 1μl of CDNPR primer (100 pmole/μl) and 1 μl of *Taq* DNA polymerase (Perkin-Elmer, 5U/μl). The tube was incubated at 94℃ for 5 min and 30 cycles of amplification was performed under the standard PCR condition of denaturing at 94℃ for 30 s and annealing at 54℃ for 30 s, extending at 72℃ for 1 min. Final extension step was performed at 72℃ for 5 min.

Analysis of RT-PCR product with restriction enzyme : The nucleocapsid gene was amplified as described above and digested by *Nco* I and *Bam* HI. The amplification was performed using clinical samples obtained from a Jindo dog, poodle dog, and badger, and PBMCs of nineteen dogs suffered from canine distemper, and five different lots of vaccines, and Lederle strain.

Nucleotide sequence analysis of partial nucleocapsid gene : Analysis of nucleotide sequence for amplified gene, a part of nucleocapsid gene of CDV was sequenced using Automatic DNA sequencer (ABI 377, Perkin-Elmer). Sequencing reaction was carried out as indicated by manufacturer. Briefly, 700 ng of DNA sample was mixed with 8 μl of ready-reaction containing each of G, A, T, and C labeled with fluorescence of different color. The reaction was added with 1 μl of *Taq* DNA polymerase (Perkin-Elmer, 5 U/μl)), 2 μl of forward or reverse primer, 2 μl of DMSO and 8 μl of D.W. PCR was performed 15 cycles under the conditions as follows: denaturing at 96℃ for 10 s, annealing at 55℃ for 5 s and extending at 70℃ for 60 s. The PCR product was precipitated and dry under vacuum. Sequencing data was analysed using DNASIS program (Hitachi, ver 7.0).

Results

Amplification of CDV gene : To compare PCR product of part of CDV NP gene, forward and reverse primers were designed. The product was amplified from nucleotide 662 to 1181 of NP gene of CDV. The clinical samples were taken from a Jindo dog, poodle dog and badger. The molecular weight of the PCR products was corresponding to 519 bp. The PCR products from the clinical samples of the dogs, badger and half of the PCR products from PBMCs showed broad band with smearing. Of 19 dogs suspected of having CDV, only 14 samples showed the nucleocapsid PCR product (Fig 1). The PCR products of five vaccines and Lederle strain showed the same band as those of the dogs and badg-

Fig 1. Amplification of partial nucleocapid gene of canine distemper virus. Clinical samples and peripheral blood mononuclear cells (PMBCs) were taken from dogs, badger and patient dogs. Total RNAs were extracted from those samples and PBMCs and then used for RT-PCR. All PCR products were run at 120V and stained with ethidium bromide. M : 1 kb ladder marker; Lanes 1-3 : clinical samples of Jindo dog, Poodle dog and badger; Lanes 4-22 : PBMCs from patient dogs.

Fig 2. Analysis of PCR products of clinical samples and vaccines with *Nco* I enzyme.
M : 1 kb ladder marker ; Lanes 1~3 : PCR products of clinical sample of Jindo dog, Poodle dog, and Badger ; Lanes 4~17 : PCR products of PBMCs of patient dogs ; Lane 18 : PCR product of Lederle strain ; Lanes 19~21 : PCR products of using vaccines in Korea ; Lanes 22~23 : PCR products of imported vaccines.

Fig 3. Analysis of PCR products of clinical samples and vaccines with *Bam* HI enzyme.
M : 1 kb ladder marker ; Lanes 1~3 : PCR products of clinical sample of Jindo dog, Poodle dog, and Badger ; Lanes 4~17 : PCR products of PBMCs of patient dogs ; Lane 18 : PCR product of Lederle strain ; Lanes 19~21 : PCR products of using vaccines produced in Korea ; Lanes 22~23 : PCR products of imported vaccines.

er (unpublished data).

Analysis of PCR product with restriction endonuclease : To see if restriction enzyme profile of PCR products of wild type CDV is different from that of vaccine strain, the PCR products of clinical samples and vaccine strains were digested with *Nco* I and *Bam* HI. The PCR products derived from seventeen clinical samples, the five vaccine strains and Lederle strain were separated into two fragments of 375 bp and 144 bp with *Nco* I (Fig 2). However it was interesting that the PCR products of clinical samples and only one vaccine were separated into two different sizes of 317 bp and 202 bp with *Bam* HI, whereas the PCR products of another four vaccine strains and Lederle strain were undigestible with *Bam* HI (Fig 3).

Nucleotide sequence analysis of RT-PCR product : Partial CDV NP gene of clinical samples were amplified and sequenced. The sequences were analysed using DNASIS program (Hitachi, ver 7.0). The sequence from clinical sample of Jindo, poodle dog and badger were identical but shared 96.3, 96.5, 93.6 and 93.4% homology with those of Yanaka, Han95, Lederle and Onderstepoort strain, respectively. Deduced amino acid sequences of the clinical samples of the above three species shared 98.8, 99.4, 100, 100 and 99.4% homology with those of Yanaka, Han95, A75/17, Lederle and Onderstepoort strain, respectively (Fig 4 and 5).

Discussion

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Jindo     ACAGGATTGC TGAGGACCTA TCTTTGAGGC GGTTCATGGT GGCACTCATC   711
Yanaka    ********** *******T** ********** *A**T***** **********   711
Han95     ********** *******T** ********** ********** A*********   711
Lederle   ********** ********** ********** *A******** ***G******   711
Onder     ********** ********** ********** *A******** ***G******   711

Jindo     TTGGATATCA AACGATCCCC GGGGAACAAG CCTAGAATTG CTGAAATGAT   761
Yanaka    ********** ********** A********* ********** **********   761
Han95     ********** ********** A********* ********** **********   761
Lederle   *****C**** ******A*** A********* ********** **********   761
Onder     *****C**** ********** A**A****** ********** **********   761

Jindo     TTGTGATATA GATAACTATA TTGTGGAAGC TGGATTAGCT AGTTTCATCT   811
Yanaka    ********** ********C* ********** ********** **********   811
Han95     ********** ********C* ****A***** ********** **********   811
Lederle   ********** ********C* ********** ***G****** *********C   811
Onder     ********** ********C* ********** ***G****** *********C   811

Jindo     TAACTATCAA ATTTGGCATT GAAACTATGT ATCCGGCCCT TGGGTTGCAT   861
Yanaka    ********** ********** ********** *******T** **********   861
Han95     ********** ********** ********** *******T** **********   861
Lederle   ********** G********* ********** *******T** **********   861
Onder     ********** G********* ********** *******T** **********   861

Jindo     GAGTTCTCTG GAGAGTTAAC AACTATTGAA TCCCTTATGA TGCTGTATCA   911
Yanaka    *****T**C* ********** ********** ********** ****A*****   911
Han95     *****T**** ********** ********** ********** ****A*****   911
Lederle   *****T**C* ****A***** ********** *****C**** ****A*****   911
Onder     *****T**C* ****A***** ********** *****C**** ****A*****   911

Jindo     ACAGATGGGT GAAACAGCAC CGTACATGGT TATTCTGGAA AATTCTGTTC   961
Yanaka    ********** ********** ********** ********** **********   961
Han95     ********** ********** ********** ********** **********   961
Lederle   ********** ********** ********** ***CT***** **C*******   961
Onder     ********** ********** ********** ***CT***** **C*******   961
Jindo     AGAACAAATT TAGTGCAGGA TCCTACCCAT TGCTCTGGAC TTATGCTATG   1011
Yanaka    ********** ********** *********C *A******** **********   1011
Han95     ********** ********** ********** ********** ******C***   1011
Lederle   *A******** *********G ********** ********** ********C*   1011
Onder     *A******** *********G ********** ********** **********   1011
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Jindo      GGAGTCGGTG TTGAACTTGA AAACTCCATG GGAGGGTTAA ATTTTGGTAG   1061
Yanaka     *****T**** ********** ******A*** ********G* ****C*****   1061
Han95      *****T**** ********** ********** ********** ****C*****   1061
Lederle    **G**T**** ********** ********** ********** ****C***C*   1061
Onder      **G**T**** ********** ********** ********GG G***C***C*   1061

Jindo      ATCCTACTTT GATCCGGCTT ATTTCAGGCT CGGGCAAGAA ATGGTTAGAA   1111
Yanaka     ********** *****A**** ********** ********** **********   1111
Han95      ********** *****A**** ********** ********** *****C****   1111
Lederle    ***T****** **C**A**** *C*****A** ********** ********G*   1111
Onder      ***T****** **C**A**** *C*****A** ********** ********G*   1111

Jindo      GATCCGCCGG CAAAGTAAGC TCTGCACTTG CCGCCGAGCT TGGCATCACC   1161
Yanaka     ****T***** ********** ********** ********** **********   1161
Han95      ****T***** ******G*** ******T*** ********** **********   1161
Lederle    ****T***** ********** ********C* ********** **********   1161
Onder      ****T***** ********** ********** ********** **********   1161

Jindo      AAGGAGGAAG CTCAGCTAGT   1181
Yanaka     *****A**G* **********   1181
Han95      *****A**G* **********   1181
Lederle    ********** **********   1181
Onder      ********** **********   1181
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Fig 4. Nucleotide sequence alignment of partial CDV NP gene.

Clinical sampale was taken from Jindo dog and its total RNA was synthesized and PCR was performed. PCR product was sequence and analysed using DNASIS program (Hitachi, ver 7.0). Nucleotide sequences of Yanaka strain and Han95 strain were taken from Genebank (Acession no. D89014 and AJ009656). Nucleotide sequences of Lederle and Onderstepoort strain were obtained from published papers. Restriction enzyme *Nco* I and *Bam* HI site are underlined.

Within the genus *Morbillivirus* under the family *Paramyxoviridae*, seven virus species have been identified to date, which infect humans (measles virus, MV), ruminants (rinderpest virus, RPV), terresteial carnivores (canine distemper virus, CDV), marine carnivores (phocine distemper virus, PDV) or cetaceans (porpoise and dolphin morbillivirus, PMV and DMV)[14,15].

Dunkin and Laidlow reported that canine distemper is a viral disease in 1926[16]. Afterwards, the disease caused by CDV infection has been controlled for many years by the use of attenuated live vaccines[3]. Recently, however, many cases of CD have occurred in Japan, European countries and Korea[6-11,17].

In this study, we were attempted to detect CDV rapidly using RT-PCR, and differentiate currently circulating CDV in susceptible domestic and wild animals in Korea from attenuated vaccine strains by RFLP and sequencing. Using primers generated from the most conserved part of CDV NP gene, we were able to detect 14 of 19 (73.5%) PBMCs obtained from pet dogs, beagle and Jindo dog, suggesting our RT-PCR assay produced sensitive results. Previsously, Shin *et al*(1995) have demonstrated the detection of CDV NP

Strain						
Jindo	RIAEDLSLRR	FMVALILDIK	RSPGNKPRIA	EMICDIDNYI	VEAGLASFIL	271
Yanaka	**********	**********	*Y********	**********	**********	271
Han95	**********	**********	**********	**********	**********	271
A75/17	**********	**********	**********	**********	**********	271
Lederle	**********	**********	**********	**********	**********	271
Onderstepoort	**********	**********	**********	**********	**********	271
Jindo	TIKFGIETMY	PALGLHEFSG	ELTTIESLMM	LYQQMGETAP	YMVILENSVQ	321
Yanaka	**********	**********	**********	**********	**********	321
Han95	**********	**********	**********	**********	**********	321
A75/17	**********	**********	**********	**********	**********	321
Lederle	**********	**********	**********	**********	**********	321
Onderstepoort	**********	**********	**********	**********	**********	321
Jindo	NKFSAGSYPL	LWSYAMGVGV	ELENSMGGLN	FGRSYFDPAY	FRLGQEMVRR	371
Yanaka	**********	*****T****	**********	**********	**********	371
Han95	**********	**********	**********	**********	**********	371
A75/17	**********	**********	**********	**********	**********	371
Lederle	**********	**********	**********	**********	**********	371
Onderstepoort	**********	**********	*********G	**********	**********	371
Jindo	SAGKVSSALA	AELGITKEEA	QL	393		
Yanaka	**********	**********	**	393		
Han95	********F*	**********	**	393		
A75/17	**********	**********	**	393		
Lederle	**********	**********	**	393		
Onderstepoort	**********	**********	**	393		

Fig 5. Comparison of deduced amino acid sequence of partial NP gene of CDV Jindo dog isolate with that of the indicated strains above. Amino acid sequences were deduced from the nucleotide sequence of partial NP gene of CDV Jindo dog isolate, and compared with known amino acid sequences of Yanaka, Han95, A75/17, Lederle and Onderstepoort, respectively.

gene in canine PBMCs by RT-PCR using two different sets of primers[5]. According to Shin *et al*, 17 out of 32 (53.1%) cases were positive by RT-PCR[5]. The specificity of our RT-PCR assay using tissue samples was confirmed by RFLP and sequencing analysis. The PCR products obtained from all isolates and tissue samples were digested by *Nco* I. Interestingly, products derived from field cases were digested by *Bam* HI, whereas 4 out of 5 vaccine strains were not digested, suggesting that our RT-PCR and RFLP assay may differentiate field strains from vaccine strains. Indeed, the partial nucleotide sequences of CDV NP gene in all field cases contained *Bam* HI restriction site, whereas the unique *Bam* HI restriction site was not found in most attenuated vaccine strains except modified Synider Hill strain (imported vaccine), supporting the consistency of our RT-PCR and RFLP assay results. At this moment, it is not clear whether the retainment of *Bam* HI restriction site in the partial NP gene relates to viral virulence since *Bam* HI restriction site was conserved in the modified Synider Hill strain. Further studies with wide variety of virulent CDV isolates are needed to determine the significance of the *Bam* HI restriction site in the partial NP gene.

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