Molecular Identification of a Recent Type of Canine Distemper Virus in Japan by Restriction Fragment Length Polymorphism

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ABSTRACT. Restriction fragment length polymorphism analysis was used to differentiate recent field viruses of canine distemper virus (CDV) from vaccine strains. Virus genomes were amplified by using reverse transcriptase-polymerase chain reaction in part of the haemagglutinin gene. After digestion with *Eco*RV, the PCR products of recent field isolates were cut into two fragments that differ from the uncut form of old strains including all of vaccine strains. This method could be applied to fresh or stored brains, spleens and peripheral blood mononuclear cells of infected dogs. This molecular approach is useful for determining the causative agent of post-vaccinated CDV infection. — KEY WORDS: canine distemper, haemagglutinin, RFLP, RT-PCR, vaccine.

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Canine distemper (CD) has been controlled during the past several decades by attenuated live virus vaccines. However, outbreaks of CD have recently occurred in Japan [7, 8, 12, 13, 16]. Similarly, during the last decade, many outbreaks of CD have been reported in several parts of the world [1–4, 9, 15, 17]. Phylogenetic analyses revealed that the recent field isolates of canine distemper virus (CDV) make a cluster which is separate from the vaccine strains, and also suggested the presence of different lineages of CDV in the world [4, 9, 10, 12].

Post-vaccinal inclusion body encephalitis has been observed in a few cases after vaccination with modified live virus vaccine [5, 11]. Sequence analysis is therefore needed for concluding whether CDV infection which occurred in vaccinated dogs was the result of infection with wild strains or infectious recovery of vaccine viruses [6, 9]. In this study, we used restriction fragment length polymorphism (RFLP) analysis after amplification of the H gene by reverse transcriptase-polymerase chain reaction (RT-PCR) with RNA from tissue and blood materials of CDV-infected dogs.

MATERIALS AND METHODS

Cells and viruses: Vero cells and B95a cells [14] were cultivated in Eagle's minimum essential medium with 5% calf serum and in RPMI 1640 with 5% fetal calf serum, respectively.

In this study, we used five laboratory CDV strains, the Onderstepoort, YSA-TC, From D, FXNO and Snyder Hill strains, which are used as vaccine strains in Japan, and nine field isolates, the MD77 (isolated in Kumamoto prefecture in 1977), DKC6 (Tokyo, 1984), Hamamatsu (Shizuoka, 1993), Yanaka (Tokyo, 1994), Adachi (Tokyo, 1995) [7, 18], Jujo (Tokyo, 1996) and Hamanako (Shizuoka, 1997) strains. The latter two strains were newly isolated from peripheral blood mononuclear cells (PBMCs). The laboratory strains and the MD77 and DKC6 strains were propagated in Vero cells, while the other field isolates were propagated in B95a cells.

Experimentally and naturally CDV-infected Dogs: Two specific-pathogen-free beagle puppies were experimentally infected with the Yanaka strain intracerebrally or intranasaly and intravenously, and then euthanized at seven days post inoculation (dpi). Five dogs were diagnosed as CDV-infected by veterinary clinicians. One of them (Dog No. c94037) died from the infection and the other four (c94071, c97006, c97007 and c97023) were euthanized at the moribund stage. Fifty to 100 mg of brains and spleens or PBMCs from these experimentally or naturally infected dogs were used for RNA extraction. PBMCs were separated from heparinized whole blood on Ficoll-paque (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) [12]. In addition to extracting RNAs from PBMCs, we attempted isolation of CDV as described previously [13]. All of the naturally infected dogs were diagnosed as having CD by clinical signs and histopathological studies.

RT-PCR and RFLP analysis: Total RNA was extracted from the virus-infected cells, tissues or PBMCs using ISOGEN (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. RT-PCR was performed using *Bca*BEST™ RNA PCR Kit (TaKaRa, Siga, Japan). Random 9 mer was used for the RT reaction and two primer sets, CDVH17 and CDVH16, and CDVH15 and CDVHd6 [12], were used for PCR. One μg of total RNA was subjected to the RT reaction according to the manufacturer's instructions, and then to denaturation and extension at 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min for 30 cycles for infected culture cells or for 50 cycles for tissues and PBMCs,

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followed by a 10 min extension step at 72°C.

Aliquots of 10 μl of the PCR products were directly digested with *Xho*I, *Ssp*I and *Eco*RV (Boehringer Mannheim GmbH, Germany). Restriction fragments were detected by electrophoresis in 1% agarose gels with ethidium bromide staining.

RESULTS

Selection of the restriction enzyme for RFLP: Based on the restriction maps of the H genes of the Onderstepoort and Yanaka strains, we selected three enzymes, *Xho*I, *Ssp*I and *Eco*RV, as candidates for RFLP analysis. We first tested five laboratory strains, the Onderstepoort, YSA-TC, From D, FXNO and Snyder Hill strains, all of which are used as vaccine strains in Japan, since the sequence of the H gene of the latter four strains have not been reported. We amplified cDNA fragments of the H genes of the five laboratory strains and one recent field isolate, the Yanaka strain, by RT-PCR with the primer set CDVH17 and CDVH16 [12]. PCR products of the expected size (1690 bp) were detected for all strains (Fig. 1). We then digested the amplified genes with the three restriction enzymes. *Xho*I digested only the Onderstepoort strain but not the other stains, so that it could not distinguish the Yanaka strain from vaccine strains. *Ssp*I digested the Yanaka strain and also the YSA-TC strain, although the RFLP pattern of the YSA-TC strain was clearly distinguishable from that of the Yanaka strain. *Eco*RV did not digest any of the vaccine strains but did digest the Yanaka strain. Therefore, we chose *Eco*RV for the following experiments.

RFLP analysis of PCR products from in vitro specimens: To obtain a larger amount of amplified products, we used another primer set, CDVH15 and CDVHd6, for shorter fragments, from nt 288 to nt 1013, of the H gene of the Yanaka strain. After digestion with *Eco*RV, amplified cDNAs from six field isolates (the Yanaka, DKC6, Hamamatsu, Adachi, Jujo, and Hamanako strains) showed 363 bp fragments (Fig. 2). Moreover, we attempted this assay to other two isolates, the Yokohama1 (Kanagawa, 1987) and Ueno (Tokyo, 1992) strains, isolated in our laboratory and one isolate, the KDK1 strain (Ibaraki, 1991; kindly provided by Kyoritsu Shoji Laboratories, Ibaraki, Japan). The RFLP pattern of them showed also the same pattern as those of six recent field isolates (data not shown). None of the laboratory strains was digested by the *Eco*RV treatment, as expected. Therefore, *Eco*RV was considered to be the most efficient enzyme for distinguishing between vaccine and recent wild strains. One old field isolate, the MD77 strain, was observed as the undigested form. This result indicates that during the past 20 years, nucleotide mutation of the H genes of recent field isolates caused the gain of the *Eco*RV site at nt 649.

RFLP analysis of PCR products from in vivo specimens: The applicability of this RT-PCR-RFLP assay for confirmation after autopsy of the infection by recent wild CDV strains was first examined using experimentally

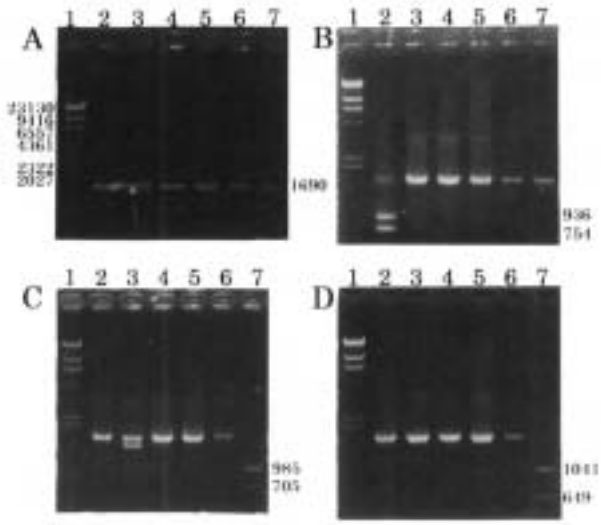

Fig. 1. Restriction enzyme patterns of CDV H genes. Lane 1, *Hin*d III digested λDNA as molecular markers. Lanes 2 to 6 are laboratory strains, the Onderstepoort, YSA-TC, From D, FXNO and Snyder Hill strains, respectively. Lane 7 is a field isolate, the Yanaka strain. A: non-digested RT-PCR products of CDV H genes. PCR products were digested with each enzyme (B: *Xho*I, C: *Ssp*I, D: *Eco*RV) and analyzed by electrophoresis in 1% agarose gels with ethidium bromide staining.

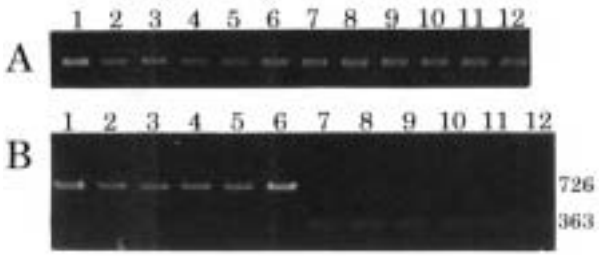

Fig. 2. Comparison of electromobilities of the RFLP patterns of laboratory strains and field isolates. Lanes 1 to 5 are laboratory strains, the Onderstepoort, YSA-TC, From D, FXNO and Snyder Hill strains, respectively. Lanes 6 to 12 are field isolates, the MD77, DKC6, Hamamatsu, Yanaka, Adachi, Jujo and Hamanako strains, respectively. Non-digested cDNA (A) and cDNA digested by *Eco*RV (B) were analyzed by electrophoresis in 1% agarose gels with ethidium bromide staining.

infected dogs (Table 1). Two dogs (Dog No. 1, 2) were experimentally infected with the Yanaka strain, and the RT-PCR products using the primer set CDVH15 and CDVHd6 were obtained from brains and spleens at seven dpi. The digestion products obtained with *Eco*RV clearly showed the same pattern as that of recent field isolates in agarose gel electrophoresis. We then used the RT-PCR-RFLP analysis on tissue specimens from five dogs which were recently affected by CD in the field. We extracted total RNA from brains and spleens stored at −80°C (dogs No. c94037 and c94071) or obtained freshly (dogs No. c97006, c97007 and

Table 1. RT-PCR-RFLP analysis of experimentally and naturally infected dogs

Experimentally infected dogs

Dog No.	Infection		Euthenasia	RELP analysis	
	Material	Route		Brain	Spleen
1	Yanaka strain	i.c.[a)]	7 dpi	W[c)]	W
2	Yanaka strain	i.v.+i.n.[b)]	7 dpi	W	W

Naturally infected dogs

Dog No.	Year	Vaccine history	Infectious phase	RFLP analysis			Virus isolation
				Brain	Spleen	PBMC	
c94037	1994	+	Acute	W	W	NT[d)]	–
c94071	1994	+	Subacute	W	W	NT	–
c97006	1997	+	Acute	W	W	W	+
c97007	1997	+	Acute	W	W	–	–
c97023	1997	–	Acute	W	NT	NT	–

a) i.c.: Intracerebral. b) i.v.+i.n.: Intravenous and intranasal. c) W: The pattern of recent field isolates. d) NT: Not tested.

c97023), or from fresh PBMCs (dogs No. c97006 and c97007). After 50 cycles of PCR, all of the RFLP patterns of the tissue specimens were clearly identified as those of recent CDV field isolates. From one of two PBMCs that was succeeded virus isolation, RT-PCR products were amplified and the RFLP pattern was simillar with those of tissue specimens.

DISCUSSION

Recently, the number of dogs affected by CD has increased in Japan [7, 8, 12, 13, 16]. Among affected dogs, vaccinated dogs have been included [7, 16]. The widespread usage of vaccines has led to the need for some practical, simple and rapid methods to distinguish between old strains including all of vaccine strains and recent field isolates.

In this study, the cDNA fragments were effectively amplified without nonspecific products from both laboratory and field isolate strains by RT-PCR with primer sets for the H gene. The restriction enzyme *Eco*RV digested all nine strains of recent field isolates but not laboratory and old field isolate, MD77, strains. Therefore, we conclude that RT-PCR-RFLP with *Eco*RV analysis is useful for rapid differentiation between old strains including all of vaccine strains and recent field isolates.

In addition, the RFLP assay could also be applied to organ specimens from affected dogs. All RT-PCR products derived from brains and spleens of experimentally infected and naturally affected dogs were well amplified and their RFLP patterns corresponded to those of recent field isolates. Organs from two dogs which were stored at −80°C for three years could be used in this assay. Thus, this assay with brain or spleen is suggested to be useful in postmortem diagnosis to confirm infection with recent wild strains of CDV, rather than infectious recovery of vaccine viruses. Since we could detect a recent field isolate pattern from PBMCs of a naturally affected dog, this method is also useful in the early stage of CD.

The mutation of C to A at nt 650 caused the difference of RFLP patterns after digestion with *Eco*RV between old strains including all of vaccine strains and recent field isolates. Since only one old strain, the MD77 strain that was isolated in 1977, showed the same RFLP pattern as vaccine strains, this mutation from C to A seems to have been made in the past 20 years.

Recently, CDV has become widespread in much of the world, and the H gene sequences of many recent field isolates have been analyzed. Based on sequence data in GenBank or EMBL databases, we searched for the *Eco*RV site in various recent isolates, 1493/Han89 (X84999), 5804/Han90 (X85000), Mink/DK86 (Z47759), Dog/GR88 (Z47760), Dog/DK91, B+C (Z47761), Dog/US89 (Z47762), Leopard/US91 (Z47763), Javelina/US89 (Z47764), Raccoon/US89 (Z47765), 404 (Z77671), 2544 (Z77672) and 4513 (Z77673) [4, 9, 10, 15]. None of them has the *Eco*RV site analyzed in this study. This means that foreign isolates could not be distinguished from vaccine strains by our assay, and that the mutation at nt 650 in the H gene is unique to recent Japanese field isolates. These findings are in agreement with the suggestion that recent field isolates of CDV can be subdivided into phylogenetic clusters reflecting their geographical origin [4, 9, 10, 12].

The RFLP patterns from all of nine recent field viruses isolated during the last 20 years and five naturally CDV-infected dogs in this study showed the recent type. Therefore, at least major population of prevalent CDV in Japan seems to be the recent field isolate type, although the possibility of existence of the old RFLP type-viruses in the field can not be excluded. If old type of RFLP pattern would be obtained from affected dogs with vaccine histories, further analyses with other methods should be needed for determining whether the infection was resulted by infectious

recovery of vaccine viruses, old type-viruses in the field, or foreign strains.

This is the first report describing a method which enables rapid discrimination between old strains including all of vaccine strains and recent field isolates in Japan. Thus, this RT-PCR and RFLP analysis of the CDV H gene will be a valuable asset for diagnosis of this virus infection.

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