Acta Veterinaria Hungarica 52 (1), pp. 125–132 (2004)

DETERMINATION AND PHYLOGENETIC ANALYSIS OF CANINE DISTEMPER VIRUS IN DOGS WITH NERVOUS SYMPTOMS IN TURKEY

A. ÖZKUL[1*], A. ARDA SANCAK[2], E. GÜNGÖR[1] and I. BURGU[1]

[1]Department of Virology and [2]Department of Internal Medicine,
Faculty of Veterinary Medicine, Ankara University, Dıskapı, 06110 Ankara, Turkey

(Received February 12, 2003; accepted April 1, 2003)

In the present study, canine distemper virus (CDV) was investigated in 20 dogs having nervous signs arousing the clinical suspicion of canine distemper (CD). A total of 13 animals (65%) were stray dogs and had no accurate record about the vaccination history. Clinical examinations revealed that the majority (85%) of the animals showed systemic form characterised by predominantly nervous symptoms accompanied by mild respiratory system signs whilst the remaining cases (15%) recorded mainly respiratory distress. CDV RNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) only in 45% of the suspected cases. Phylogenetic analysis of partial nucleotide sequence of the P gene coding region revealed that the virus is closely related to European strains. Immune responses in 13 cases (65%), which were detected by dot-ELISA, indicated inefficient levels for neutralising functions against CDV. It was postulated that this response could have been mediated by either previous vaccination or mild infection with field strains.

Key words: Canine distemper, nervous symptoms, RT-PCR, dot-ELISA

Canine distemper virus (CDV) is a member of the genus Morbillivirus of the Paramyxoviridae family, and has been known for centuries to cause severe systemic disease throughout the world. The virus is a highly contagious and devastating pathogen (Blixenkrone-Moller, 1993; Pringle, 1999). CDV infection may result in subclinical disease, a catarrhal form manifesting itself in gastrointestinal and/or respiratory signs, and a systemic form characterised by fever, rhinitis, and respiratory problems frequently associated with central nervous system involvement (Appel and Gillespie, 1972; Appel, 1987). Encephalomyelitis is the most common cause of death of CDV-infected animals (Vandevelde and Zurbriggen, 1995). The onset of encephalitis in animals appears to be influenced by humoral immune responses to CDV (Rima et al., 1987). Dogs that have demyelinating lesions, CDV proteins and infectious virus in their brains demonstrate

[*]Corresponding author; E-mail: ozkul@veterinary.ankara.edu.tr; Phone: +90.312.3170315 Ext: 364, 255; Fax: +90.312.3164472

0236-6290/$ 20.00

an impaired immune response. During later phases of the disease, appearance of antibodies against the H protein of the virus correlates with the absence of lesions, CDV antigens and infectious virus in the brains of these dogs (Rima et al., 1991).

Animals infected with CDV shed the virus in the discharges from their nose and eyes and through their saliva, urine and faeces (Appel, 1987; Willis, 2000). Various specimens including blood smears, skin biopsy specimens, conjunctival and vaginal smears, and cerebrospinal fluid taps have been used for diagnosis of the disease (Alldinger et al., 1993; Baumgartner, 1993).

Despite extensive vaccination with attenuated live strains of CDV, distemper suspect cases in young dogs are being reported in Turkey, especially in certain types of breeding units where the dogs are grouped in kennels, stray pounds and pet shops. In the present study, canine distemper was diagnosed by using reverse transcription-polymerase chain reaction (RT-PCR), and the results were correlated to clinical signs and the humoral response against CDV in Ig level obtained by a commercial dot-ELISA system (ImmunoComb®, Biogal Galed Labs, Israel) in 20 dogs with nervous symptoms. In addition, phylogenetic analysis of the virus based on the partial P gene sequences was performed in order to get some ideas about the origin of the virus detected.

Materials and methods

Altogether 20 dogs with nervous signs arousing the suspicion of canine distemper, which had been brought to the clinics of the Veterinary Faculty of Ankara University between February and December 2001, were included in this study. Individual data (age, sex, breed and vaccination history) of the animals are listed in Table 1. The animals were divided into two categories according to the clinical symptoms they demonstrated, i.e. systemic and catarrhal forms. In general, the majority (n = 17) of the animals displayed the systemic form characterised by fever, mucopurulent rhinitis and conjunctivitis, respiratory dysfunction and severe neurological alterations including seizures, rhythmic tonic and clonic convulsions and mild chewing actions in the mandible. Two of the animals (No. 1 and 15) showed the catarrhal form including a variety of gastrointestinal and respiratory signs. In one animal (No. 20), severe chronic pododermatitis resistant to medical therapy was also present (Table 1).

Swab samples were taken from both nostrils and eyes, and immediately immersed into TE buffer for transport and short-term storage. Nasal and ocular swabs were used as a source of RNA for RT-PCR detection of viral genome. CDV RNA was extracted from swab samples using the technique described by Chomczynski and Sacchi (1987). Complementary DNA synthesis and subsequent DNA amplification was carried out as described by Gassen et al. (2000) in 10 µl and 30 µl total volumes, respectively. A panmorbillivirus-specific primer

set was selected from the P protein-coding gene sequence. Designed forward (sense) and reverse (antisense) primers are as follows: UPPF - 5'-ATGTTTATGATCACAGCGG-3' (position in CDV 2132>2150 nt), UPPR - 5'-ATTGGGTTGCACCACTTGCT-3' (position in CDV 2542<2560 nt).

Table 1

Individual and clinical properties of CD-suspected cases and the results of RT-PCR and dot-ELISA

Dog No.	Breed	Sex	Age (months)	Origin [1]	Vaccine [2]	Clinical findings [3]	RT-PCR [4]		dot-ELISA [5]
							NS	CS	
1	Kangal	M	8	H	NV	C	+	–	2+
2	Mixed	M	6	S	NV	S	+	–	3+
3	Kangal	M	9	H	V	S	–	+	5+
4	Kangal	M	9	H	V	S	–	–	5+
5	Husky	F	3	H	NV	S	+	+	1+
6	Mixed	F	4	S	UK	S	–	–	3+
7	GSD	M	3	H	V	S	–	–	6+
8	Mixed	F	7	S	UK	S	+	–	4+
9	Kangal	M	9	S	NV	S	–	–	2+
10	Mixed	F	5	S	UK	S	+	–	2+
11	Husky	M	4	H	UK	S	–	–	6+
12	Mixed	F	12	S	UK	S	+	–	2+
13	Mixed	F	5	S	UK	S	–	–	6+
14	Mixed	F	6	S	UK	S	–	–	6+
15	Mixed	F	12	S	UK	C	–	–	3+
16	Mixed	F	12	S	UK	S	–	+	2+
17	Mixed	F	6	S	UK	S	–	–	6+
18	Mixed	F	10	S	UK	S	+	–	3+
19	Mixed	M	5	S	UK	S	–	–	2+
20	GSD	M	18	H	NV	P	–	–	3+

[1]H: Household dog; S: Stray dog; [2]V: Vaccinated; NV: not vaccinated; UK: unknown; [3]C: Catarrhal; S: Systemic; P: Pododermatitis; [4]NS: Nasal swab; CS: Conjunctival swab; [5]Numbers indicate antibody level calculated using the colour-coded scale provided with the kit

Using this pair of primers, it was expected to amplify a 429-bp DNA product. The DNA products were sequenced directly using a T7 polymerase-based commercial kit (Pharmacia). Analysis of sequence data was carried out using the GCG (Genetics Computer Group Inc., Madison, WI) package. Nucleic acid sequences obtained from PCR products were aligned with known sequences from representatives of CDV, and a phylogenetic tree was generated with the DNADIST and KITSCH programmes of the PHYLIP 3.5 software (Felsenstein, 1989).

Blood samples collected by venipuncture from the jugular vein were clotted and the serum samples obtained were tested for the presence of CDV-specific antibodies by using a commercially available dot-ELISA system (Immuno-Comb®, Biogal Galed Labs, Israel) as described by the manufacturer. The con-

centration of CDV antibodies in each sample was measured by the use of a colour-coded scale provided in the kit, and translation of the colour reaction to CDV IgG antibody units was carried out expressing between 0 to 6 as described in the user's manual.

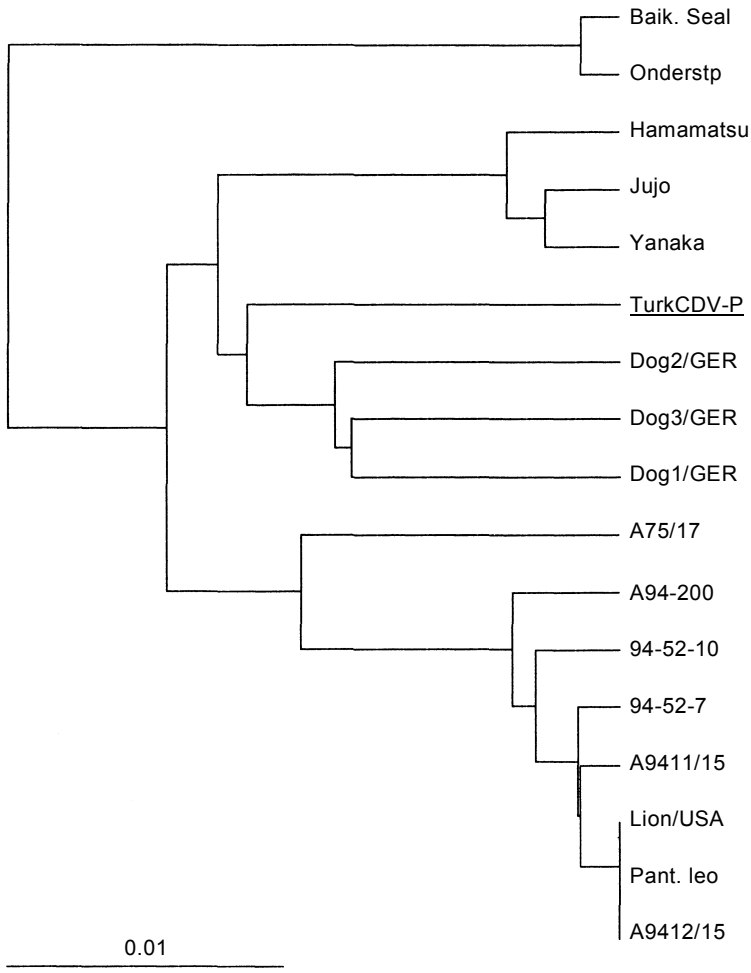

Fig. 1. Phylogenetic analysis of the partial nucleotide sequence of the CDV P coding gene. The tree is based on neighbourhood-joining method and was derived by using theDNADIST and KITSCH programmes of PHYLIP 3.5 software. Branch lengths are proportional to the genetic distances between viruses and the hypothetical ancestor at the nodes in the three. Sequences extracted from the database for the following strains (nucleotide sequence accession numbers are given in parenthesis): TurkCDV-P (AF384686), Dog1/GER (AF259549), Dog2/GER (AF259550), Dog3/GER (AF259551), A75/17 (AF164967), Baikal Seal (AF181446), Hamamatsu (AB028915), Yanaka (AB028914), Jujo (AB028916), A9412/15 (U53711), 94-52-10 (U53712), 94-52-7 (U53713), A94-200 (U53714), A9411/15 (U53715), Lion/USA (U76708), *Panthera leo* (Z46431), Onderstepoort (AF305419). The bar represents nucleotide substitutions per position

Results

The P gene-coding region was partially amplified by RT-PCR in swab samples of 9 (45%) dogs in the expected size for the primer set indicated before. In 6 of these animals, amplicons were obtained from nasal swabs and in only two animals from ocular swab samples (Table 1). One animal (No. 5) revealed positive amplification in both swab samples. Partial nucleotide sequence of the phosphoprotein (P) coding gene region was submitted to GenBank (TurkCDV-P, Accession number AF384686). Figure 1 shows the inferred phylogenetic relationship between the viruses detected in this study and other CDVs. The field CD viruses detected in this study were found to be more closely related to those reported in Europe in comparison to CDVs isolated from other geographical regions such as Germany.

Using the commercial dot-ELISA system, +3 and higher (as detected by the colour pattern) CDV-specific antibodies were found in 13 (65%) animals. Antibody response that was highly efficient (+4 and above) was detected in 8 (61%) animals while the remaining five (39%) were shown to have less efficient (+3) antibody level against the virus (Table 1).

Discussion

The present work is the first report on the laboratory confirmation of CD in dogs with nervous symptoms in Turkey. A total of 20 CD-suspected cases were involved in the study. CDV RNA was detected by RT-PCR in 45% of the suspected cases. The dot-ELISA revealed that 65% of the dogs had previous exposure to the virus.

In this study, dogs suspected of having CD showed predominantly nervous symptoms accomplished with increased nasal and ocular secretions rather than gastrointestinal or respiratory signs. Furthermore, in the animals examined in this study, breed and sex did not seem to have any effects on the occurrence of the disease as described in previous reports on the recent CDV outbreaks in Japan (Gemma et al., 1995; Gemma et al., 1996).

There are numerous reports concerning the use of RT-PCR for the diagnosis of CDV infection in surviving animals (Shin et al., 1995; Frisk et al., 1999; Mochizuki et al., 1999), and researchers have described various types of samples depending on the nature of the clinical signs. In the present study, an RT-based PCR technique was used for the detection of CDV RNA in nasal and ocular swab samples collected from cases characterised by nervous disorders. The RNA of the virus was detected in nasal and/or ocular swabs of as few as 9 (45%) cases, which is in agreement with the results of Frisk et al. (1999). This might be related to either a lower copy number of viral particles secreted in the nasal or

ocular mucosae, loss of CDV RNA by autolytic changes in epithelial cells after collection or to the efficacy of the primers as discussed by Frisk et al. (1999). On the other hand, one should remember that CDV RNA might uncommonly be found in serum and blood samples that were not used in this study, from animals with subacute or chronic distemper encephalitis (Frisk et al., 1999). The phylogenetic analysis of P gene product from RT-PCR revealed that CDV detected in this study was found to be more closely related to European rather than vaccine, Asian and American strains of the virus.

Serological monitoring results indicated previous contacts to either wild or attenuated strains of CDV. As previously mentioned by Waner et al. (1998), a 5+ and higher antibody titre is accepted as to be protective against CDV. In animal No. 3, however, the antibody titre (5+) estimated by the ruler within the test kit did not seem to have a protective effect against virus replication and secretion from the body. Thus, it was concluded that the antibody response detected by the dot-ELISA might not correspond to neutralising activity against field viruses. On the other hand, the dot-ELISA might be advantageous in terms of quantity of the antibody response detected. Therefore, knowledge of the approximate antibody level may help to determine the immune status to CD acquired actively (i.e. through vaccination or subclinical disease) or passively (i.e. by the transfer of maternal antibodies).

The other interesting point is that animal No. 3 in which CDV RNA was detected by RT-PCR has been reported by its owner as having received a series of vaccines against CD. As has been pointed out before, the antibody response, which was not verified by virus neutralisation test (VNT), does not always correlate with the level of protective immunity. Previously, immunity to challenge exposure with virulent CDV was shown to last as long as 7 years, even though serum antibody titres decreased while older, previously vaccinated dogs have been reported to have contracted CD and died (Appel and Gillespie, 1972). However, this argument is not sufficient to justify the situation in case No. 3, since the dogs involved in this study were all younger than 18 months. The most speculative explanation for case No. 3 is to implicate the failure of the vaccine administered.

In conclusion, canine distemper is caused by Europe-related CD virus in Turkey and is predominantly seen in stray dogs. The disease is characterised mainly by severe nervous symptoms and mild respiratory signs. These animals play a virus reservoir role for housed animals. Apart form stray dogs, occurrence of the disease was detected in previously vaccinated animals, which might have been caused either by ineffective vaccines and/or vaccinations or by escape mutants that can infect the animal in spite of vaccinal protection. Further molecular analysis of surface glycoproteins (e.g. H and F coding regions) of the circulating virus will help us understand the surface topology which interacts with the immune system and target cells, so that mechanisms for neurovirulence and escape from humoral immunity can be revealed.

Acknowledgement

This study was supported by a grant from the Biotechnology Institute of Ankara University (No. 28/2002). The authors wish to thank to Dr Thomas Barrett, Institute for Animal Health, Pirbright Laboratory, UK, for providing panmorbillivirus P gene specific primers used in the RT-PCR.

References

Alldinger, S., Baumgartner, W., Van Moll, P. and Örvell, C. (1993): *In vivo* and *in vitro* expression of canine distemper viral proteins in dogs and non-domestic carnivores. Arch. Virol. **132,** 421–428.

Appel, M. (1987): Canine distemper virus. In: Appel, M. J. G. (ed.) Virus Infections of Carnivores. Elsevier Science Publishers, Amsterdam, The Netherlands. pp. 133–159.

Appel, M. and Gillespie, J. H. (1972): Canine distemper virus. In: Gard, S., Hallauer, C. and Meyer, K. F. (eds) Virology Monographs 11. Springer-Verlag, New York, N.Y.

Baumgartner, W. (1993): Virale Infektionskrankheiten bei Welpen und Junghunden unter besonderer Berücksichtigung der Staupevirusinfektion. Prakt. Tierarzt **1,** 26–32.

Blixenkrone-Moller, M. (1993): Biological properties of phocine distemper virus and canine distemper virus. Acta Path. Microbiol. Immun. Scand. [Suppl.] **101,** 1–51.

Chomczynski, P. and Sacchi, N. (1987): Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Ann. Biochem. **162,** 156–159.

Felsenstein, J. (1989): PHYLIP – Phylogeny inference package. Cladistics **5,** 164–166.

Frisk, A. L., König, M., Moritz, A. and Baumgartner, W. (1999): Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. J. Clin. Microbiol. **37,** 3634–3643.

Gassen, U., Collins, F. M., Duprex, W. P. and Rima, B. K. (2000): Establishment of a rescue system for canine distemper virus. J. Virol. **74,** 10737–10744.

Gemma, T., Miyashita, N., Shin, Y. S., Okita, M., Mori, T., Iwatsuki, K., Mikami, T. and Kai, C. (1995): Serological survey of canine distemper virus infection using enzyme-linked immunosorbent assay. J. Vet. Med. Sci. **57,** 761–763.

Gemma, T., Watari, T., Akiyama, K., Miyashita, N., Shin, Y. S., Iwatsuki, K., Kai, C. and Mikami, T. (1996): Epidemiological observations on recent outbreaks of canine distemper in Tokyo area. J. Vet. Med. Sci. **58,** 547–550.

Mochizuki, M., Hashimoto, M., Hagiwara, S., Yoshida, Y. and Ishiguro, S. (1999): Genotypes of canine distemper virus determined by analysis of the hemagglutinin genes of recent isolates from dogs in Japan. J. Clin. Microbiol. **37,** 2936–2942.

Pringle, C. R. (1999): Paramyxoviridae. Arch. Virol. **144 (Suppl. 2),** 421–429.

Rima, B. K., Baczko, K., Imagawa, D. T. and Ter Meulen, V. (1987): Humoral immune response in dogs with old dog encephalitis and chronic distemper meningo-encephalitis. J. Gen. Virol. **68,** 1723–1735.

Rima, B. K., Duffy, N., Mitchell, W. J., Summers, B. A. and Appel, M. J. (1991): Correlation between humoral immune responses and presence of virus in the CNS in dogs experimentally infected with canine distemper virus. Arch. Virol. **121,** 1–8.

Shin, Y. S., Mori, T., Okita, M., Gemma, T., Kai, C. and Mikami, T. (1995): Detection of canine distemper virus nucleocapsid protein gene in canine peripheral mononuclear cells by RT-PCR. J. Vet. Med. Sci. **57,** 439–445.

Vandevelde, M. and Zurbriggen, A. (1995): The neurobiology of canine distemper virus infection. Vet. Microbiol. **44,** 271–280.

Waner, T., Naveh, A., Meir, N. S. B., Babichev, Z. and Carmichael, L. E. (1998): Assessment of immunization response to canine distemper virus vaccination in puppies using a clinic-based enzyme-linked immunosorbent assay. Vet. J. **155,** 171–175.

Willis, A. M. (2000): Canine viral infections. Veterinary Clinics of North America. J. Small Anim. Pract. **30,** 1119–1133.