

1-1-2010

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SHABBIR, MUHAMMAD ZUBAIR; RABBANI, MASOOD; AHMAD, ARFAN; AHMED, ALİ; MUHAMMAD, KHUSHI; and ANWAR, IKRAM (2010) "Comparative evaluation of clinical samples from naturally infected dogs for early detection of canine distemper virus," *Turkish Journal of Veterinary & Animal Sciences*: Vol. 34: No. 6, Article 11. <https://doi.org/10.3906/vet-0808-16>  
Available at: <https://journals.tubitak.gov.tr/veterinary/vol34/iss6/11>

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## Comparative evaluation of clinical samples from naturally infected dogs for early detection of canine distemper virus

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Received: 11.08.2008

**Abstract:** Early detection of canine distemper (CD) is critical for appropriate and timely treatment. The present study included 45 suspected cases with respiratory signs for a comparative evaluation of clinical samples in the early detection of canine distemper virus (CDV). Thirty-four dogs exhibited digestive system involvement, but none showed any neurological symptoms. Clinical progression of CD, based on age, sex, vaccination status, disease, and clinical findings, was observed. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect viral nucleoprotein RNA in plasma, conjunctival and nasal swabs. Only 10 dogs were positive for CDV infection. Among the 45 dogs, CDV RNA was detected in 5, 6, and 10 dogs using plasma, nasal and conjunctival swabs, respectively. The results suggest that the incidence of CD is independent of age and may involve secondary infections. For early detection of CDV, conjunctival swab is the most reliable sample ( $P < 0.05$ ), as compared to nasal swab and plasma.

**Key words:** Canine distemper, RT-PCR, conjunctival swab.

Canine distemper (CD) is a systemic infection associated with high morbidity and mortality (1). Susceptibility to this infection largely depends on the immunodeficient status of the host (2). The disease is most commonly observed in puppies, at a time when they have lost their maternally derived antibodies (3). This may result in the simultaneous or sequential appearance of subclinical infection, gastrointestinal and/or respiratory, neurologic, and cutaneous lesions (2,4). Neurological signs may be present in chronic form, with or without other clinical manifestations (5).

The prevalence of CDV, based on clinical cases, has been reported to be 11% in Pakistan (6). The high cost of the vaccine, vaccine failure, and lack of awareness about the importance of vaccination, as well as the lack of a national pet registration policy and laboratory-based disease diagnostic facilities are some of the factors that result in perpetuation of the CDV in Pakistan.

Subclinical or mild cases of CDV infection are not commonly diagnosed (3). Clinical signs similar to other respiratory and enteric diseases of dogs concurrent with CDV further complicate the clinical

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diagnosis of CD (5). However, in certain cases such symptoms as conjunctivitis, purulent ocular and nasal discharge, respiratory catarrh, fever, and diarrhea are strongly suggestive of CDV infection (2,3). Although laboratory-based confirmation is required, due to the distribution of the virus in different parts of the body and tissues (4), various specimens, such as whole blood, conjunctival and nasal swabs, urine, and CSF, can be used for diagnostic purposes (3,4). Among these, plasma or serum (4), and conjunctival and nasal swabs (3) are most commonly used for viral detection (2-4,7). Various laboratory procedures used to detect the CDV antigen, such as cell culture, the ferret inoculation test, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, and in situ hybridization, are laborious and time consuming (3,4).

Comparatively, reverse transcriptase polymerase chain reaction (RT-PCR) is a more rapid diagnostic test for detection of CDV infection (3), though its sensitivity varies with the nucleic acid extraction method and primers used for amplification of the target region (8,9). Due to the contagious nature and high mortality of CD, quarantine of infected dogs is necessary to prevent the disease from spreading. For proper treatment and patient management accurate diagnosis of CD infection at an early stage is essential. The aim of the present study was to use RT-PCR to evaluate the suitability of various fluid samples from dogs with respiratory distress for early pre-mortem diagnosis.

During 2007, 3 sample types from each of 45 suspected dogs were collected. All the dogs had fever and respiratory system involvement, such as purulent ocular and nasal discharge, conjunctivitis, bronchitis, and bronchopneumonia. A log was maintained on the basis of age, sex, disease progression, body temperature, and medical treatment. The dogs ranged in age from 2 months to 3 years. Most of the dogs (68.88%) were less than 18 months old. In all, 55.7% of the dogs were male and 42.23% were female.

From each of the 45 dogs suspected to be infected with CDV, 1 mL of peripheral whole blood from the cephalic vein was collected in a vacutainer (Venoject, Belgium), and conjunctival and nasal swabs were obtained by swabbing with sterilized cotton sticks in a microfuge tube with 0.5 mL of sterilized 0.9% saline

solution. The blood was centrifuged at 1000 ×g for 10 min to collect plasma. In addition, the same number of samples were taken from each of 5 non-vaccinated healthy dogs as a negative control, while commercially available multivalent vaccine (Hexadog, Merial, France) served as a positive control. Nasal and conjunctival swabs were stored at -20 °C until further use. All the samples were collected at the University of Veterinary and Animal Sciences (UVAS) Pet Center and private clinics in Lahore.

Viral RNA of CD was extracted from plasma (200 µL), conjunctival swab (200 µL), and nasal swab (200 µL) using One Step RNA Reagent (Bio-Basic Inc., Canada). Briefly, cells were lysed with TriZole (1:4) containing guanidine isothiocyanate and phenol, followed by phase separation with chloroform (200 µL). The RNA present on the top aqueous phase was precipitated by adding an equal volume of isopropyl alcohol (500 µL). Total RNA was extracted by centrifugation at 12000 ×g, followed by washing the pellet with 75% (1000 µL) and 100% ethyl alcohol (1000 µL), and subsequent drying at room temperature. The extracted RNA was dissolved in DEPC water (20 µL) and used for reverse transcription using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, EU). One microliter of random hexamers (0.2 µg/µL) and 4 µL of DEPC water were added to extracted RNA in equal volume. This was followed by addition of 1 µL of RiboLock™ (20 U/µL), 4 µL of 5× reaction buffer, and 1 µL of 10 mM dNTPs, followed by incubation at 25 °C for 5 min. To this 1 µL of M-MuLV reverse transcriptase (200 U/µL) was added and a thermocycler (Thermo Electron, Finland) was set up for 1 cycle each, as follows: incubation at 25 °C for 10 min, 42 °C for 1 h, and 70 °C for 10 min. The synthesized cDNA was chilled by placing the microfuge tubes on ice and stored at -20°C until further use.

The complete nucleoprotein coding genome region of CDV RNA was amplified using the primer pair described by Yoshida et al. (10), with minor modification. The oligonucleotide (e-oligos, GeneLink, USA) primers used were a 21mer (sense) (5'GTGTCAGAAATAGCATCCAAG3', nt 1287-1307) and a 26mer (antisense) (5'GTGGGATCCAGACTGGTCTTGAATAT3', nt 1705-1680). PCR amplification was performed in a

total volume of 25  $\mu$ L after addition of 5  $\mu$ L of extracted cDNA, 2  $\mu$ L (10 pmol/ $\mu$ L) of each primer pair, 12  $\mu$ L of PCR master mix (Eppendorf, Germany), and 6  $\mu$ L of DEPC water. The thermocycler was set up as follows: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 30 s each at 94 °C, 59 °C, and 72 °C. Amplification was terminated by a final extension at 72 °C for 10 min. The amplicons were resolved on 1.5% agarose gel containing 0.5  $\mu$ g/mL of ethidium bromide, following electrophoresis at 3.5 V/cm. The bands were observed under UV light and documented photographically.

In the present study the comparative suitability of various biological fluid samples obtained from dogs naturally infected with CDV was evaluated. CDV infection was suspected in these dogs based on such clinical signs as conjunctivitis, bronchopneumonia, serous, and purulent nasal discharge. Some dogs exhibited digestive system involvement, including vomiting and diarrhea, along with secondary infections, but all suspected dogs were devoid of any neurological symptoms (Table). Clinical decisions were made and medical treatment was administered by the attending veterinarian at the UVAS Pet Center and at private clinics.

Table 1. Brief history, clinical findings, CDV NP gene detection by RT-PCR and disease outcome in dogs naturally infected with CDV.

Dog ID	Age (Months)	Fever (°C)	Sex	Vaccination record	Disease progression (days before)	Clinical findings	Secondary infections	RT-PCR results using			Disease outcome clinically
								P	NS	CS	
3/07	13	40.2	<sup>a</sup> M	<sup>e</sup> V	7	<sup>c</sup> Respiratory and <sup>d</sup> Digestive system involvement	E. coli and Toxocara canis	-	+	+	Recovered
11/07	21	39.3	M	V	2	Respiratory system involvement	<sup>h</sup> N	-	-	+	Recovered
12/07	9	40.9	M	<sup>f</sup> NV	NI	Respiratory and Digestive system involvement	Ancylostoma caninum and Babesia spp.	-	+	+	Death showing <sup>l</sup> Nervous symptoms
21/07	17	39.4	<sup>b</sup> F	<sup>g</sup> NI	NI	Respiratory system involvement	N	+	+	+	Recovered
23/07	4	39.3	M	NV	3	Respiratory and Digestive system involvement	Toxocara canis	-	+	+	Recovered
31/07	7	40.1	F	V	11	Respiratory and Digestive system involvement	Dipylidium caninum	+	+	+	Recovered
32/07	13	40.6	F	NI	8	Respiratory and Digestive system involvement	Uncinaria spp.	+	-	+	Recovered
33/07	16	40	M	NV	NI	Respiratory and Digestive system involvement	Salmonella spp.	-	+	+	Death
41/07	9	39.5	M	NV	3	Respiratory system involvement	Toxocara canis and Ancylostoma caninum	+	-	+	Recovered
43/08	9	39.6	F	V	7	Respiratory system involvement	Babesia spp.	+	-	+	Recovered

<sup>a</sup>M Male; <sup>b</sup>F Female; <sup>c</sup>Respiratory system fever coughing serous or mucopurulent nasal discharge dyspnea and conjunctivitis;

<sup>d</sup>Digestive System anorexia depression dehydration vomiting and diarrhea; <sup>g</sup>NI no information; <sup>f</sup>NV not vaccinated; <sup>e</sup>V vaccinated;

<sup>h</sup>N no secondary infection; <sup>l</sup>Nervous symptoms muscle twitching ataxia of limbs disorientation and in-coordination in body movements; P Plasma; NS Nasal swab; CS Conjunctival swab.

The clinical plasma, and conjunctival and nasal swab samples were processed for definitive diagnosis through RT-PCR, which resulted in amplicons of the expected length (419 bp) for the primer pair described by Yoshida et al. (10) (Figure 1). Only 10 (22.22%) of the 45 samples were positive for CDV infection (Figure 2). Among the 10 distemper-positive dogs, CDV RNA was detected by RT-PCR in 50% of the

plasma samples, 60% of the nasal swabs, and 100% of the conjunctival swabs, versus 11.11%, 13.33%, and 22.22%, respectively, from the total of 45 suspected dogs (Figure 2). No amplicons of expected length were obtained from clinically healthy dogs (negative control). The RT-PCR results obtained from the various biological fluids and disease outcome are presented in the Table.

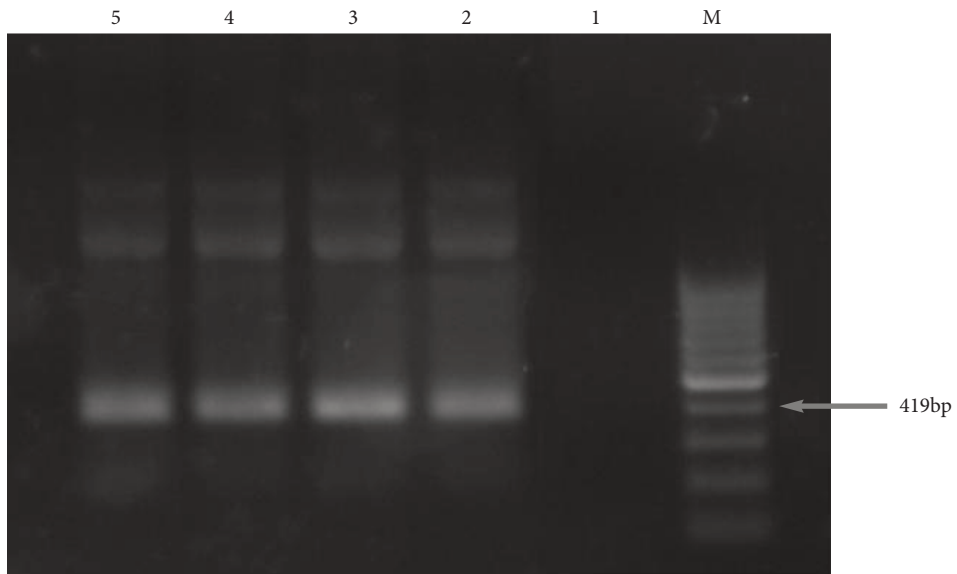
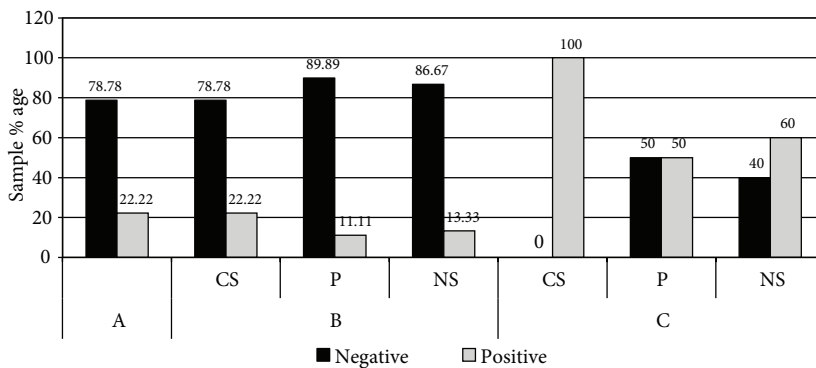


Figure 1. Electrophoresis of RT-PCR product from CDV infected dogs. Lane M, Molecular size marker Gene Ruler 100bp DNA; Lane 1, Negative Control; Lane 2, Positive Control with Hexadog vaccine; Lane 3, Conjunctival swab; Lane 4, Nasal swab and Lane 5, plasma.



A = Detection percentage of CDV from all dogs  
 B = Comparative detection percentage of clinical specimens within all dogs  
 C = Comparative detection percentage of clinical specimens within distemper positive dogs

Figure 2. Comparative detection percentage of CDV amplicons RT-PCR using conjunctival swab (CS), nasal swab (NS), and plasma (P).

The diagnostic value derived from clinical specimens was insignificant ( $P = 0.306$ ) in all suspected cases, according to the chi-square distribution test; however, it was significant ( $P = 0.034$ ) in distemper-positive dogs. The detection rate of expected CDV amplicons from conjunctival swabs was significantly higher than that from the other samples used during the study.

The severity of CD depends on the virulence of the viral strain, environmental conditions, and the susceptibility of each animal (2,11). After rabies, CD is the most fatal disease of dogs worldwide. The prognosis of CDV infection is guarded in all animals and poor in susceptible animals, especially after the onset of neurological symptoms. The virus takes 10-14 days to cross the blood-brain barrier (12); therefore, early and accurate diagnosis of CDV infection is important for reducing mortality by using immune serum to prevent the virus from entering the brain.

In recent years RT-PCR has been proven to be a fast, sensitive, and specific method for the detection of CDV (8,13). The set of primers we employed has been successfully used by other researchers with high specificity and sensitivity (10). The clinical utility of tissue and fluid samples varies, depending on the onset of infection (3). Sera are reported to be highly sensitive when suspected dogs exhibit respiratory and digestive system involvement (4,9). The virus has been detected in secretions from conjunctiva and nasal epithelium 1-14 and 3-14 days post-infection, respectively (3), indicating them suitable early diagnostic specimens for antigen detection. In urine, the presence of the virus occurs later (5-14 days post-infection) (3). It is possible for the virus to enter the brain before it enters the urine; however, signs of the chronic stage or nervous system involvement make it of good diagnostic value.

In the present study the viral genome was detected in 10 of the 45 suspected CD cases. The remaining 35 dogs were negative. This supports similar clinical findings in other respiratory and enteric diseases of dogs, including canine hepatitis, canine parvovirus, corona virus, herpes virus, parainfluenza, and leptospirosis. Moreover, the sensitivity of RT-PCR depends on the selection of primers used for amplification of the target position in the viral genome (4), as well as the nucleic acid extraction

method and nature of the sample (8). The viral genome was not detected in all of the 10 distemper-positive dogs using serum and nasal swabs. This may have been due to the presence of neutralizing antibodies and the lack of or low level of CDV RNA (4). Appel (14) observed the gradual disappearance of CDV antigen in dogs that developed antibodies as a result of experimental infection.

The viral genome was detected in all the distemper-positive dogs using conjunctival swabs. It has been reported that the virus appears early and is eliminated later from conjunctival fluid than any other fluid sample (3). During the early phase of CD the conjunctiva and eyes become infected at the time of generalized viremia (15). Additionally, CDV replicating in the conjunctival sac or orbital cavity is not subject to immunity, avoiding rapid elimination (3). There is also widespread ocular involvement in CDV-infected conjunctival epithelium, corneal epithelium, and the iris (3).

Among the infected dogs, 1 male and 1 female died, despite routine care and supportive treatment administered by the veterinarian during the study period. Both were admitted with a history of respiratory and digestive system involvement. Later, the male dog showed neurological signs, such as ataxia, seizures, and uncoordinated body movements, whereas these symptoms were absent in the female. Although progression of the disease could not be determined in these 2 dogs, it was assumed that the virus had entered the nervous system before supportive medical treatment was administered. The virus can invade the central nervous system (CNS), resulting in a demyelinating disease approximately 10 days post-infection (2). The extent of neuropathological changes is highly influenced by the age of the dog, immunocompetence at the time of infection, and the viral strain (11). This may be the reason why RT-PCR results using serum samples were negative in dogs showing neurological symptoms (3,13).

Nearly all the distemper-positive dogs developed secondary infections (Table), which may have been due to CDV-induced immunosuppression caused by virus-mediated T and B cell cytolysis, and dysfunction

of CD4<sup>+</sup> T cells (11). It has also been shown that depletion of cytokine-producing cells or virus-induced down-regulation of cytokine production leads to minimal peripheral blood cytokine production, resulting in reversible massive cytolytic virus-induced immune suppression (11). This condition can persist for several weeks and is responsible for susceptibility of animals to opportunistic infections (16).

In conclusion, the present study indicates that in the absence of a history of trauma, dogs with conjunctivitis, nasal discharge, fever, and diarrhea should be considered highly suspicious for CD,

regardless of other clinical signs. It supports previous reports of the usefulness of RT-PCR as a fast, sensitive, and specific diagnostic tool for pre-mortem detection of CD (4). Additionally, conjunctival swabs are the most useful in the early diagnosis of CD in naturally infected dogs, followed by nasal swabs and plasma.

### Acknowledgements

We thank Dr. Susan Baigent (Institute for Animal Health, Compton, UK) and Abdul Waheed Hashmi (UVAS, Pakistan) for reviewing and editing the article.

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