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The Investigation of Canine Distemper Virus in Different Diagnosis Materials of Dogs using Molecular and Pathological Methods, Northeastern Turkey

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ABSTRACT

Background: *Canine distemper virus* (CDV) is highly contagious disease that affects dogs despite several control measures. This study was aimed at investigating the presence of CDV nucleic acid in different clinical and tissue materials, from naturally infected dogs, by reverse transcriptase-polymerase chain reaction (RT-PCR) and to molecularly characterize distemper strains according to the partial Nucleoprotein (NP) gene sequence. Furthermore, tissue samples under went histopathological examination for distemper infection.

Methods: A total of 202 different diagnosis materials were collected from dogs (n=60) in the Kars region in northeastern Turkey. The samples were tested for CDV using RT-PCR with primers designed for the CDV NP gene. Samples determined as positive for CDV (n=7) were sequenced. Tissue samples underwent histopathological examination.

Result: Most of the cases were in animals aged 0-6 months. The most common clinical finding was severe respiratory system infection. This finding was accompanied by gastrointestinal and nervous system infections. CDV nucleic acid was detected in 112 of 202 materials by RT-PCR. According to RT-PCR results, positivity rates of 88.2% (30/34), 72.2% (13/18), 60% (3/5), 55.5% (10/18), 55.5% (10/18), 51.6% (16/31), 45.5% (5/11), 37.8% (14/37) and 36.7% (11/30) were detected in nasal swab, lung, footpad, kidney, spleen, rectal swab, cerebrospinal fluids (CSF), leuokocyte and cerebellum samples, respectively. Viral nucleic acids were detected at higher rates in nasal swabs. The phylogenetic assessment of the amplicon sequences revealed a 97.7%-100% similarity among the Turkish CDV strains, which are independent from vaccine strains, were found to be more closely related to the European lineage. Intranuclear and intracytoplasmic inclusion bodies were detected by histopathology. This is the first study to investigate CDV in naturally infected dogs from northeastern Turkey and to provide novel and updated epidemiological information.

Key words: *Canine distemper virus*, Molecular characterization, RT-PCR.

INTRODUCTION

Canine distemper (CD) is a major viral infection commonly observed in domestic and wild carnivores (McCarthy *et al.* 2007). The infection is very common worldwide and has high transmission and mortality rates, especially in immunologically compromised populations. In 1906, Carré determined that the causative agent of this disease, known since the 16th century, is a virus (Loots *et al.* 2017). *Canine distemper virus* (CDV), belonging to the *Morbillivirus* genus in the *Paramyxoviridae* family, has a very close genetic and antigenic relationship with other members of this genus (Mochizuki *et al.* 1999, von Messling *et al.* 2001). The viral genome has a total of 6 gene regions, namely, the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (H) and polymerase protein (L) gene regions (Kodi *et al.* 2021). The H protein is one of the two surface proteins of the CDV and allows the virus to attach to cellular receptors. Moreover, the H protein is the major determinant of viral tropism and cytopathogenicity. Based on phylogenetic analyses of the H gene, CDV strains cluster in 12 different lineages, namely, America 1, America 2, Asia 1, Asia 2, Europe/South America 1, Europe wildlife, South America 2 and 3, Arctic, Rockborn-like, Africa 1 and Africa 2 (Bhatt *et al.* 2019). The N protein, found in both the virion and infected cell, is involved in shaping the viral genome and protecting the nucleic acid. Furthermore, the N protein is involved in viral replication and transcription (Lee *et al.* 2010). The target of the CDV is

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lymphoid tissue and mucous membrane cells throughout the body. Due to viral affinity, some respiratory, digestive and central nervous system cases develop an acute, subacute or chronic infection accompanied by skin rashes and hyperkeratosis (Mochizuki *et al.* 1999, Deem *et al.* 2000).

Even if clinical findings suggest CDV infection, a definitive diagnosis requires laboratory testing. A number of testing methods are used to diagnose CDV infection. The ELISA test is widely used in serological studies (Gençay *et al.* 2004, Kelly *et al.* 2005). Virus isolation (Maclachlan and Dubovi, 2011), immunohistochemical and immunofluorescence assays (Çomakli *et al.* 2020, Zhang *et al.* 2020), sandwich-dot enzyme-linked immunosorbent assay (dot-ELISA) (Li *et al.* 2013, Zhang *et al.* 2020) and in situ hybridization (Zurbriggen *et al.* 1993) have been used for the detection of the CDV antigen. However, most of these methods are laborious, time-consuming and not very productive. Today, the methods frequently used to diagnose CDV infection are the rapid test, RT-PCR and nucleotide sequencing (Maganga *et al.* 2018; Oguzoglu *et al.* 2018; Costa *et al.* 2019; Çomakli *et al.* 2020, Zhang *et al.* 2020, Kodi *et al.* 2021). Molecular methods can be performed only in equipped laboratories, but are highly sensitive and useful. Organ materials such as lymph nodes, the spleen, liver and lungs as well as oral and nasal swabs, serum, whole blood and CSF samples can be used in PCR tests to identify CDV nucleic acid (Frisk *et al.* 1999; Shin *et al.* 2004; Lan *et al.* 2006; Kim *et al.* 2006; Saito *et al.* 2006).

This study was aimed at investigating the presence of CDV nucleic acid in different clinical samples and organ materials, from naturally infected dogs, by RT-PCR and to molecularly characterize distemper strains according to the partial N gene sequence. Furthermore, tissue samples underwent histopathological examination for distemper infection.

MATERIALS AND METHODS

Ethics statement

This research was conducted pursuant to the approval of the Animal Testing Local Ethics Council of Kafkas University (Approval Number: KAU HADYEK-2019-006).

Sampled animals and sample collection

The dogs sampled in this study were obtained from Kafkas University Veterinary Faculty, Clinics and Pathology Department between 2020 and 2021 in the Kars region of Turkey. In total, 60 dogs of varying age and breeds, showing signs of high fever, respiratory, digestive and/or nervous system infections, were sampled. A total of 202 materials (37 leuokocyte, 34 nasal and 31 rectal swab samples, 30 cerebellum, 18 lung, 18 kidney, 18 spleen, 11 CSF and 5 footpad) were collected from dogs with clinically suspected distemper. 9 out of 60 dogs were vaccinated and the other dogs were unvaccinated.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted from the samples using a High Pure Viral RNA Kit (Roche, Mannheim, Germany) and complementary DNA (cDNA) synthesis was performed using a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific, USA), according to the manufacturers'instructions. RT-PCR was performed using the method and primer (PP-1) described by Frisk *et al.* (1999). The formation of PCR products of the expected size (287 bp) was analysed by DNA gel electrophoresis. The primer sequences used in this study are shown in Table 1.

Sequencing and phylogenetic analyses

Seven PCR products with clean and good PCR bands as a result of RT-PCR were subjected to sequence reaction via service procurement (DNA Lab, İstanbul, Turkey). The raw sequencing data were aligned using BioEdit version 7.0.5 (Hall, 1999) with the Clustal W algorithm. The phylogenetic map of the aligned sequences was established using MEGA version 6.0 (Tamura *et al.* 2013). Partial genome sequences of CDV, deposited in GenBank under the following accession numbers, were used: MH496779 (CDV6_TH/2014), KJ466106 (CDV_SY), EU716337 (164071), KJ123771 (17139113), AY386315 (5804), MF437053 (Gabon), MF041963 (CDV06), AF378705 (Ondersteport), GU138403 (SnyderHill/Vaccine), KJ747371 (13_1941), KJ747372 (13_2262), KX774415 (PT61/Pt/2004), KF914669 (CDV2784/2013), KY971532 (WT02SA), KY971528 (W01SA), MT149210 (CDV-CP8-TH/2017), MH496777 (CDV8_TH/2014), AB474397 (007Lm), AB475097 (M25CR), MH484613 (Kiki), KM280689 (Uy251) (Fig 1).

Histopathology

For histopathological examination, tissue samples were taken at systemic necropsies, fixed in 10% buffered formaldehyde solution, embedded in paraffin wax, sectioned at 5 µm and stained with Mayer's haematoxylin-eosin (H&E). The sections were examined and photographed under a light microscope.

RESULTS AND DISCUSSION

Clinical findings

In this study, 46 out of 60 suspicious animals were evaluated as positive by PCR.

The evaluation of the distribution of CDV infection by age, in 4 different age groups, revealed 23 cases (50%) in the 0-3 months age group, 12 cases (26%) in the 3-6 months age group, 10 cases (21.8%) in the 6-12 months age group and 1 case (2.2%) in the group over 12 months of age.

No reliable information could be obtained on the vaccination status of the vaccinated cases (completed or single dose), as either the animals were abandoned at the clinic or the animal owners were not informed.

Based on anamnesis and clinical findings, 4 cases (8.7%) had respiratory system infection and another 4 cases

(8.7%) had nervous system infection alone. Some animals had developed multisystemic infections. Infections of the respiratory and digestive systems in 7 cases (15.2%), respiratory and nervous systems in 11 cases (24%), digestive and nervous systems in 11 cases (24%) and respiratory, digestive and nervous systems in 9 cases (19.6%) were found to coexist. Skin lesions characterized by rash along with systemic infection findings were detected in 5 cases (11%). In these cases, hyperkeratosis had developed on the footpad and tip of the nose.

RT-PCR

CDV nucleic acid was detected in 112 of 202 materials by RT-PCR with PP-I primer pairs. The 287 bp-DNA product of CDV was observed in all positive samples. According to RT-PCR results, positivity rates of 88.2% (30/34), 72.2% (13/18), 60% (3/5), 55.5% (10/18), 55.5% (10/18), 51.6% (16/31), 45.5% (5/11), 37.8% (14/37) and 36.7% (11/30) were detected in the nasal swab, lung, footpad, kidney, spleen, rectal swab, CSF, leuokocyte and cerebellum samples, respectively.

Sequencing and phylogenetic analyses

Seven samples yielded 287 bp-amplicons upon RT-PCR with NP gene specific primers. The amplicons were sequenced and their GenBank accession numbers are TR-KARS-CDV-1 (MW921485), TR-KARS-CDV-2 (MW921486), TR-KARS-CDV-3 (MW921487), TR-KARS-CDV-4 (MW921488), TR-KARS-CDV-5 (MW921489), TR-KARS-CDV-6 (MW921490) and TR-KARS-CDV-7 (MW921491). The strains obtained in this study were 97.7-100% similar to each other and 94.5-98.1% similar to the reference strains. According to the phylogenetic tree, the strains identified in this study showed a higher affinity to the European strains (MF437053 and AY386315), but were genetically distant to the vaccine strains (AF378705 and GU138403) (Fig 1).

Table 1: Nucleotide sequence and position of primer pairs used for RT-PCR.

Primer code	Nucleotide position	Nucleotide sequences (5′-3′)	Product size (bp)
p1	769-789	ACA GGA TTG CTG AGG ACC TAT	287
p2	1055-1035	CAA GAT AAC CAT GTA CGG TGC	

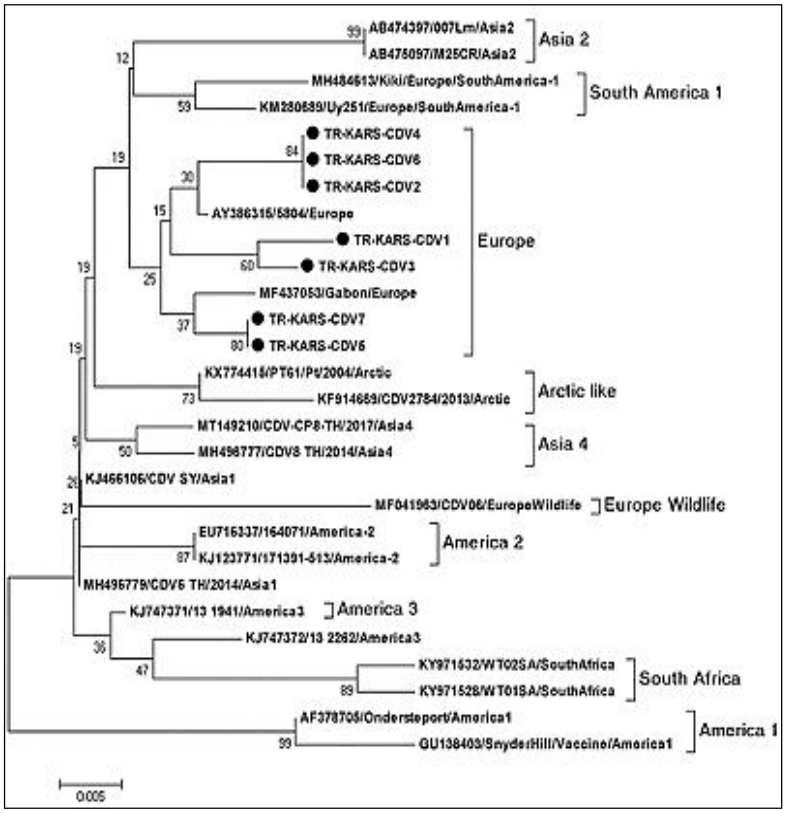

Fig 1: Phylogenetic tree of CDV strains all groups N gene partial sequences. Sequence dataset was analysed using MEGA 6.0, the neighbour-joining (NJ) method and bootstrap analysis (1,000 replicates) based on the Clustal W algorithm. Significant bootstrapping values are shown on the nodes. (•) Turkish CDV strains are indicated by a black round sign and all Turkish strains detected in Europe lineage.

Histopathology

Macroscopic results

Hyperkeratosis was detected on both sides of the nose and on footpads in Dogs 38, 43, 46, 52 and 53. The lungs were not collapsed, hyperemic or edematous. The spleen and liver were enlarged and haemorrhagic. Mild catarrhal enteritis was observed, especially in the ileum. While the serosal surface of the urinary bladder had a normal structure, hyperemic areas were detected on the mucosal surface.

Microscopic results

Generally, the cases with respiratory symptoms displayed interstitial pneumonia. Mononuclear cell infiltration existed in the bronchi and bronchioles and the interalveolar septum had thickened due to lymphocyte infiltration. The most conspicuous histological findings were eosinophilic cytoplasmic and nuclear inclusion bodies. Purulent bronchopneumonia was accompanied by interstitial pneumonia in Dogs 3, 14, 26 and 45 (Fig 2 a-c). Intranuclear inclusion bodies were observed in the epithelial cells of the renal pelvis. The urinary bladder was locally hyperemic and intranuclear and intracytoplasmic inclusion bodies were detected in the mucosal epithelial cells (Fig 3 a-b). While demyelinated areas were absent in the pons and medulla oblangata, necrosis and neuronophages were observed in the neurons. Significant demyelination was detected in the cerebellum, near the 4th ventricle and cerebral peduncle. Also, demyelinated areas were seen in different regions of the subtantia alba in the cerebellum. Intranuclear eosinophilic inclusion bodies existed in astrocytes localized to the demyelinated areas. Perivascular mononuclear cell infiltrations were detected in the cerebellum and brain stem (Fig 4 a-d).

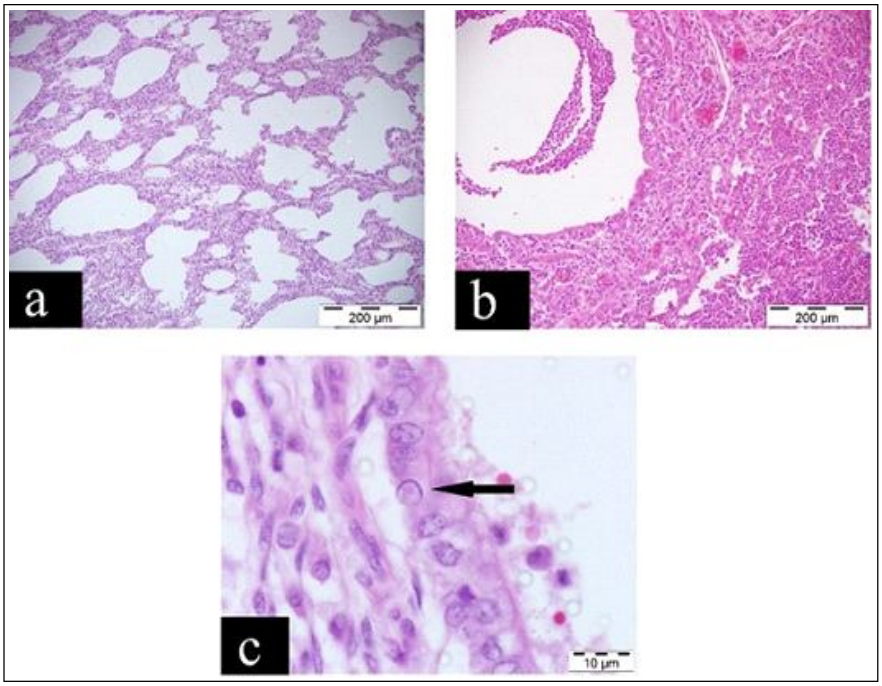

Fig 2: a) Interstitial pneumonia in a dog infected with CDV. H&E, 10X, b) Purulent bronchopneumonia, secondary infection, H&E, 10X, c) Lung. Acidophilic intranuclear inclusion bodies in bronchial epithelial cells of dog lung (arrows). Mononuclear cell infiltration around the bronchiole. H&E, 120X.

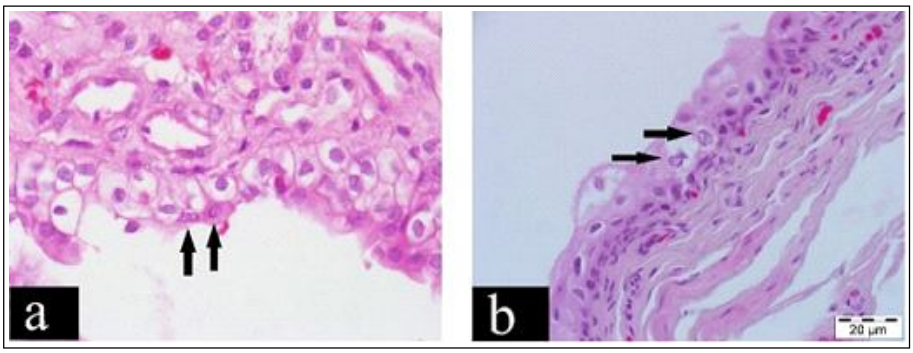

Fig 3: a) Kidney intranuclear inclusion bodies in pelvis renalis epithelium (arrows), H&E 80X, b) Urinary bladder. Intranuclear and intrastoplasmic inclusion bodies (arrows), H&E, 60X.

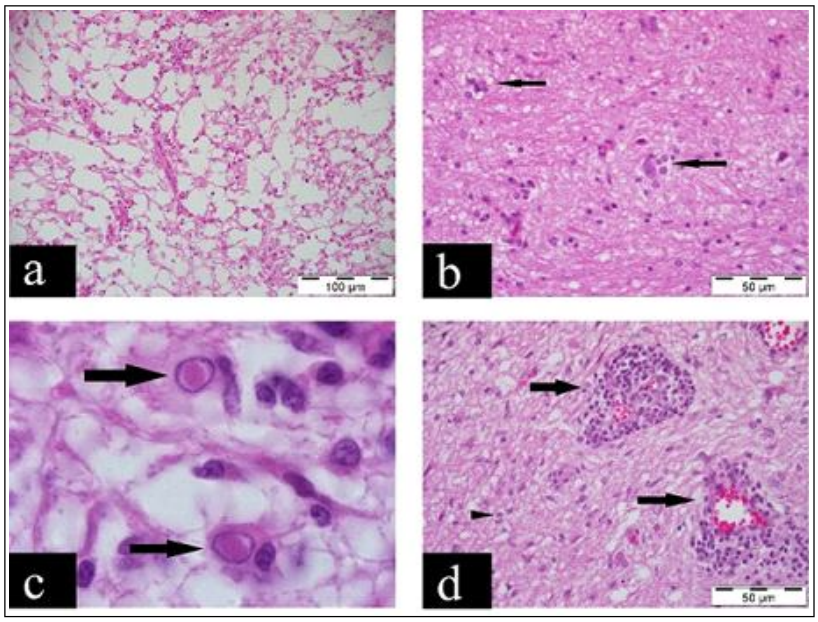

Fig 4: a) Demyelination areas in the cerebellum, H&E, 20X, b) Neuronophagia in the brain stem (arrows), H&E, 40X, c) Cerebellum, intranuclear inclusion bodies in astrocytes (arrows), H&E, 200X, d) Cerebellum, perivascular mononuclear cell infiltration in the 4th ventricle (arrows), intranuclear inclusion body in astrocytes (arrowhead), H&E, 40X.

RT-PCR, used to diagnose CDV infection, is a fast and reliable diagnostic method. This viral nucleic acid assay can be applied to samples of both living and dead animals, regardless of the form of the disease, immune response and viral antigen distribution (Frisk *et al.* 1999). RT-PCR is a fast and sensitive method that allows the detection of small amounts of viral RNA even in the advanced stages of the disease (Barrett, 1999). PCR's increased effectiveness depends on many factors, such as the number of cycles, quality of the starting material, length of the target DNA and variability of the annealing and elongation temperatures. Using RT-PCR, Frisk *et al.* (1999) detected CDV RNA in 25 of 29 (86%) serum samples and 14 of 16 (88%) whole blood and CSF samples from 38 dogs with clinically suspected distemper. In the present study, materials including nasal swabs, lungs, footpads, kidneys, spleens, rectal swabs, CSF, leuokocytes and cerebella were used to diagnose CDV infection with RT-PCR. The materials that were used were of high diagnostic value. Positivity rates for nasal swabs, lungs, footpads, kidneys, spleens, rectal swabs, CSF, leuokocytes and cerebella were 88.2% (30/34), 72.2% (13/18), 60% (3/5), 55.5% (10/18), 55.5% (10/18), 51.6% (16/31), 45.5% (5/11), 37.8% (14/37) and 36.7% (11/30), respectively. Viral nucleic acid was detected at higher rates in the nasal swabs, compared to the other samples. The high positivity rates detected in the nasal swabs and the respiratory system infection findings observed were consistent with each other. Kim *et al.* (2006) evaluated the presence of viral nucleic acid in nasal and conjunctival swabs, leukocytes and urine samples with RT-PCR in the early stages of the disease in dogs experimentally infected with CDV. They reported that nucleic acid was detected 1-14 days after infection in the conjunctival swabs and 3-14 days after infection in the nasal swabs, at varying levels. Lan *et al.* (2005) determined that viral nucleic acid could be determined with RT-PCR in nasal swabs of dogs experimentally infected with CDV, up to the 28th day post-infection. Kim *et al.* (2006) reported that the detection of viral nucleic acid in leukocyte samples was more difficult and less practical, because the virus is eliminated from the blood faster than from other tissues.

In agreement with the report of Murphy *et al.* (1999), CD infection was most common in animals aged 0-6 months in this study. Recent studies on cellular receptors used by the CDV suggest that especially the infection of young animals may be related to the virus-specific receptors in the immune system (Tatsuo *et al.* 2001).

The most common clinical findings were respiratory system infection accompanied by cough and nasal and ocular discharge. In parallel, a high positivity rate was detected in nasal swabs with RT-PCR. Anamnesis revealed that a short-term gastrointestinal infection developed approximately 15 days before acute clinical infection in 5 dogs (Dogs 3, 10, 20, 26 and 45). The development of infection during this period was attributed to the secondary viral proliferation stage following viremia (Bertus and Duprex, 2006). Acute infections were associated with nervous system findings such as nervous tics, abnormal gait and chewing movements of the jaw, all resulting from CNS infections. Studies have shown that the clinical findings and pathological lesions of CNS infection vary with the CDV strain (Summers *et al.* 1984).

Dogs 38, 43, 46, 52 and 53 showed hard pad disease, coinciding with the histopathological findings of CDV

infection. Hard pad disease is a dermatological sign of CDV infection and is characterised by hyperkeratosis, a pathognomonic sign of the disease (Headley and Sukura, 2009). We detected CDV nucleic acid in the footpads of Dogs 38, 43 and 52. Although inclusion bodies were not identified within the epithelia of the footpads, CDV RNA was detected molecularly.

Macroscopic lesions were similar to those reported in previous studies. For example, lungs with foci of consolidation, as well as enlarged spleens and livers, have been reported in dogs (Viana *et al.* 2020, Headley *et al.* 2018). Microscopically, the lungs displayed interstitial pneumonia and cytoplasmic and intranuclear inclusion bodies were observed in the bronchiolar and alveolar cells. The pathological findings of the respiratory system were consistent with previous studies (Viana *et al.* 2020, Headley *et al.,* 2018). Neurological signs are common in animals with CDV and were observed both clinically and pathologically in our study. Intranuclear eosinophilic inclusion bodies existed in the astrocytes localized to the demyelinated areas. Perivascular mononuclear cell infiltrations were detected in the cerebellum and brain stem. The microscopic findings of the nervous system agreed with the results of previous studies (Klemens *et al.* 2019, Çomaklı *et al.* 2020).

Ozkul *et al.* (2004) reported that the CD viruses circulating in Turkey were similar to the European strains. Similarly, the strains detected in this study were genetically related to viruses of the European lineage. Although there are not many CDV isolates deposited in the gene bank at the N gene level, phylogenetic analysis shows that the European strains form 2 clusters. While AY386315 and TR-KARS-CDV- 1, 2, 3, 4, 6 are located in the same cluster, MF437053 and TR-KARS-CDV- 5 and 7 are located in the other cluster. Therefore, even if studied in a small geographical region, differences detected between strains in the same region are important. This points out to the necessity for more detailed molecular epidemiological studies. The strains detected in this study were only 94.5-95.4% similar to the vaccine strains and were genetically quite different from the vaccine strains which located in America 1 lineage. In this study, CDV infection was also detected in vaccinated animals. The occurrence of CDV infection in the vaccinated animal may be due to many factors (inactivaton of the vaccine virus, not being vaccinated at the right time, insufficient vaccination, *etc.*), as well as the genetic difference of the vaccine strain can be considered.

CONCLUSION

RT-PCR is very sensitive in the diagnosis of CD infection. Necropsy samples are valuable diagnostic material and nasal swabs have the highest diagnostic value in the detection of CDV with RT-PCR. Turkish CDV strains are independent from vaccine viruses and have been found to be more closely related to the European lineage. CDV infection was also detected in vaccinated animals in present study. The occurrence of CDV infection in the vaccinated animal may be due to many factors (inactivaton of the vaccine virus, not being vaccinated at the right time, insufficient vaccination, *etc.*), as well as the genetic difference of the vaccine strain can be considered.

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