

Pathological and Molecular Investigation of Canine Distemper Virus: Phylogenetic analysis of co-circulating genetic lineages based on H and F genes

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Abstract

Canine distemper virus (CDV) is a multi-systemic, highly contagious virus that infects a wide variety of carnivore species, ranging from subclinical to fatal disease. In this study, dogs with clinically suspected Canine distemper were examined by reverse transcriptase-polymerase chain reaction (RT-PCR), histopathology and immunohistochemistry. Phylogenetic analysis was performed using the obtained partial H and F gene sequences and the sequences were compared with CDV strains obtained from the GenBank. Histopathologically, characteristic intracytoplasmic and/or intranuclear inclusion bodies were observed in the CNS, lung, stomach, small intestine, liver, kidney, and spleen with encephalitis, interstitial/bronchointerstitial pneumonia, gastroenteritis. In addition, CDV antigens were detected in all tissues with characteristic histopathological findings of CDV antigens were detected in all tissues with characteristic histopathological findings of Canine Distemper. The antigens were more intense in bronchial and bronchiolar with interstitial and bronchointerstitial pneumoni and inclusion bodies, syncytial cells. It was determined with the phylogenetic map created at the end of the sequence analysis that European and Arctic strains are circulating together in the region.

Introduction

Canine Distemper Virus is widespread all over the world and causes a highly contagious, multisystemic infection (Deem et al. 2000; Martella et al. 2006). The infection characterized by respiratory and gastrointestinal, neurological signs such as fever, nasal discharge, respiratory distress, cough, conjunctivitis, anorexia, vomiting, diarrhea, lymphopenia, convulsion, seizures and muscle spasms. The virus infects canidae (domestic and wild), Procyonidae, Mustelidae, Hyaenidae, Ursidae, Viveridae and Felidae (Deem et al. 2000; Nikolin et al. 2017).

Multisystemic disease occurs as a result of replication of the virus in epithelial cells of the respiratory tract, gastrointestinal tract and urogenital system in cases where the immune response is inadequate in CDV-infected animals and this infection generally results with death. The most important transmission route of infection is the extracts and secretions of the infected animals, especially respiratory secretions. It can be spread by direct contact with infected animals or inhalation of aerosol (Deem et al. 2000; Carvalho et al. 2012).

CDV is a member of the Morbillivirus genus of the Paramyxoviridae family. It contains a single-stranded, negative sense RNA genome and sensitive to disinfectants (von Messling et al. 2001). The genome of the virus consists of six gene regions. These gene regions encode the nucleocapsid (N) protein that forms the capsid, the phosphoprotein (P) and polymerase (L) proteins that together form the replicase-transcriptase complex, the matrix protein (M) and the fusion (F) and hemagglutinin (H) proteins that facilitate the cell entry (Sidhu et al. 1993; Martella and Buonavoglia 2008). In addition, the H protein participates in the attachment of the virus to cellular receptors and is important in determining viral tropism and cytopathogenicity (Haas et al. 1999; von Messling et al. 2001). Various phylogenetic studies based on the H gene sequence from various strains of CDV identified in various geographic regions around the world

have been performed to reveal the genetic diversity of CDV. Based on H-gene sequences, CDV strains have been classified into at least 18 major genetic lineages: America-1, America-2, North America-3, South America/North America-4, America-5 (a sub-genotype of America-2), Asia 1–5, Europe Wildlife, Arctic, Africa-1, Africa-2, Europe-1/South America-1, South America 2–3 and Rock born-like (Anis et al. 2018; Bhatt et al. 2019; Duque Valencia et al. 2019; Weckworth et al. 2020).

In this study, it was aimed to determine the presence of virus in the tissues of dogs that died as a result of CDV infection by virological and immunohistochemical methods, to update the molecular information about CDV infection in our country, and to detection and molecular characterization of circulating strains based on partial H and F genes.

Materials And Methods

Samples

The material of the study consisted of tissue samples taken from 11 unvaccinated, under one year old dead dogs, showed clinically symptoms characterized by respiratory, nervous and gastroenteritis. Tissue samples were collected for histopathologic and PCR investigation. Tissues were stored frozen at -80°C until tested by PCR.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Phylogenetic Analyses

Extraction of viral RNA from tissue samples; was performed using a commercial viral nucleic acid isolation kit in accordance with the kit's procedure. RNA obtained by viral RNA extraction was used as template for complementary DNA (cDNA) synthesis. For this purpose, cDNA synthesis was performed using a commercial kit (Thermo Scientific, USA) containing the Reverse Transcriptase enzyme, in accordance with the kit's procedure. It was performed using RT-PCR method and primers specific to CDV F and H gene regions. Amplified PCR products were analyzed by gel electrophoresis. The primer sequences used in the PCR method are shown in Table 1.

The amplified PCR products were purified using a commercial purification kit and then sequence analysis was performed by the Sanger method. The raw data obtained after sequencing were aligned with the Clustal W algorithm using BioEdit version 7.0.5 (Hall 1999). Nucleotide sequences were compared with different reference Canine Distemper virus isolates using BLAST software available in the NCBI database. Phylogenetic analysis was performed with MEGA v6.0 program. For this purpose, the neighbor joining method was used. Bootstrap value calculated as 1000 iterations (Tamura et al. 2013).

Histopathology and Immunohistochemistry (IHC)

Histopathologically and immunohistochemical examinations were performed only PCR positives cases. The tissues (lung, small intestine, brain, liver, kidney, stomach, spleen, and mesenteric lymph nodes) were

fixed in 10% neutral buffered formalin, processed, and further embedded in paraffin, cut into 4 µm sections, and stained with hematoxylin and eosin (HE) using routine protocols for histopathological examination.

Immunohistochemical examination for the detection CDV antigen was performed using the avidin–biotin immunoperoxidase complex method. Sections on adhesive slides were deparaffinized and dehydrated. Peroxidase activity was blocked in 3% hydrogen peroxide–methanol solution. The sections were washed with phosphate buffered saline (PBS), and nonspecific proteins were blocked by incubating protein-blocking sera for 5 min. The sections were incubated with mouse monoclonal CDV antibody (CDV nucleoprotein, 1:400 dilution, VMRD, Pulman, USA) for 1 h at room temperature. After washing with PBS, the sections were incubated for 20 min with a biotinylated goat anti-rabbit antibody at room temperature. After another PBS rinse, the sections were treated with horseradish peroxidase–conjugated streptavidin for 20 min. After washing in PBS, DAB (Diaminobenzidine /Cell Signaling, 8090S) was used as a chromogen, and Mayer's hematoxylin was used for counterstaining. Tissue sections from a previous case of distemper were used as positive controls. For negative controls, phosphate buffered saline solution was substituted for the primary antibody

Results

CDV nucleic acid was detected by PCR in the tissues of 5 of 11 dogs that consisted the study material. PCR products obtained from different tissues of CDV infected dogs were subjected to sequence analyzes for two different gene regions, F and H. It was determined that four of the CDV sequences (TR/BAL/CDV/53, TR/BAL/CDV/54, TR/BAL/CDV/55, TR/BAL/CDV/59) obtained from the study showed high similarity among themselves (94.7% - 98.5%), they are of Arctic lineage and form a separate branch. The other sequence named TR/BAL/CDV/60 was determined to be located in a separate branch and to be in European lineage. As a result of the phylogenetic analysis created from the partial F gene sequences obtained from GenBank and the sequences obtained from our study; it was determined that four of our sequences (TR/BAL/CDV/53, TR/BAL/CDV/54, TR/BAL/CDV/55, TR/BAL/CDV/59) were close to the strains obtained from Turkey, Brazil and Germany (Fig. 1). Phylogenetic analysis result obtained from the partial H gene sequences obtained from GenBank and the sequences obtained from our study determined that it was close to strains obtained from Turkey (similarity rate; 95.8% - 97.7%) and Italy strains (similarity rate; 95.0%-97.6%). It is noteworthy that our other sequence (TR/BAL/CDV/60) is close to the South African strain (Fig. 2).

When the amino acid similarity, formed as a result of the sequence analysis with the haemagglutinin gene, was investigated, it was determined that there were differences with the strains from the GenBank (Fig. 3).

A histopathological examination of all tissues found to be RT-PCR positive for Canine distemper virus was performed. Demyelination, focal gliosis, intracytoplasmic and intranuclear inclusions were observed brain (Fig. 4a). There were mononuclear cell infiltrations around the vessels. CDV antigen was detected in

astrocytes, glia, neuron and purkinje cells the by immunohistochemically. Positive immunoreactivity more intense in areas where demyelination was observed (Fig. 4b).

In all cases, signs of interstitial pneumonia were detected in the lungs. Alveolar septa was thickened. Bronchointerstitial pneumonia was observed in three of the cases. Purulent bronchopneumonia findings were present in addition to interstitial pneumonia findings. Characteristic intracytoplasmic and intranuclear inclusions were frequently detected in bronchial/bronchiolar epithelial cells and in one case syncytial cells (Fig. 4c). In only one case, syncytial cells in the lung were significantly numerous. CDV antigen was detected in the bronchial and bronchiolar epithelial cells, alveolar macrophages, inclusions, and syncytial cells (Fig. 4d).

Lymphoid depletion and necrosis of the follicles of the spleen was observed in the case with syncytial cells in the lung. Syncytial cells (Fig. 5a) and eosinophilic intranuclear inclusions (Fig. 5b) were observed in the white pulp, especially in the necrotic areas. Eosinophilic intranuclear inclusions were particularly frequently in cells at necrotic areas (Fig. 5b). In the gastric, intestinal, and pancreatic epithelial cells, intracytoplasmic and intranuclear inclusions that immunopositivity for CDV were detected (Fig. 6a, 6b, 6c, 6d). In addition, necrosis in the crypt epithelium of the small intestinal were observed. CDV antigens were detected in all tissues with histopathological findings, consistent with the severity of the lesions.

Discussion

There are many viral agents that cause multisystemic infections in dogs (Dinçer et al. 2020; Timurkan et al. 2021). CDV infection is one of the most important of these, which is a lethal, contagious infection of carnivore species that is common all over the world and in our country. The virus, which is a multicellular pathogen, has the ability to infect three distinct types of host cells, including epithelial, lymphoid and neurological cells, and creates a generalized disease manifestation by affecting the digestive, respiratory and central nervous systems (Rendon Marin et al. 2019).

Several studies have been conducted all over the world on the genetics of canine distemper virus, including different gene regions. This study, which includes strains consisting of 208 sequences isolated from 1975 to 2011 and belonging to sixteen countries from all continents, and aimed to investigate the evolution and global distribution of the virus, was based on the H gene. It was revealed that the virus, which diversified into two ancestral clades in the 1880s and spread worldwide to form the eight of the nine current lineages. The other lineage was determined to form the North America-1 clade (Panzer et al. 2015). In different studies conducted in our country and in the world, the NP gene was chosen because it is a protected gene region and provides convenience in detecting the presence of virus (Ricci et al. 2021; Yılmaz et al. 2021; Saltık and Kale 2022). Although it is difficult to detect by PCR, phylogenetic studies are conducted with the H gene. The hemagglutinin (H) gene shows high heterogeneity. At the same time, it has been revealed that 549 amino acids of the protein encoded by the H gene may have a direct role in determining the host tropism of the virus (Bhatt et al. 2019). In addition, it is widely used to determine the relationship between CDV strains due to its key role in the formation of genetic diversity, cell tropism, host

variability and protective immunity (Mira et al. 2018, Aziz et al. 2020). Therefore, both the F gene and the H gene were selected in present study.

At least 18 major genetic strains have been identified to date in studies of the molecular characterization of the virus. Asia-1 strains are present in many countries such as China, Thailand, and Korea and show a wide geographic distribution (Radtanakantikanon et al. 2013; Bae et al. 2013). A new genetically distinct CDV strain (India -1/Asia -5) has been identified in India (Bhatt et al. 2019). On the other hand, the Asia-2 strain is determined to be circulating in Pakistan (Aziz et al. 2020). Studies have reported that South American strains, which form four different lineages, show a wide variety (Panzera et al. 2014).

Arctic strains were identified in the late 1980s. At that time, these lineages were determined to be responsible for the deaths of seals in northwestern Europe and Siberia (Likhoshway et al. 1989; Visser et al. 1990). Since then, the existence of Arctic lineages has been reported in various parts of the world and in many European countries (Italy, Hungary, China, Iran, Turkey) and America (Demeter et al. 2007; Namroodi et al. 2015; Mira et al. 2018; Nguyen et al. 2021; Koç et al. 2021). Molecular data of CDV were revealed for the first time in the west of Turkey with our study and it is seen that strains belonging to the Arctic lineage still circulate predominantly in our country.

Studies have shown that both European and Arctic strains are circulating in many countries (Italy, Hungary, Iran) (Demeter et al. 2007; Namroodi et al. 2015; Ricci et al. 2021). It has been determined that European and Arctic strains circulate together in Iran, where vaccination is limited to certain regions. Considering the geographical location of our country with Iran, the circulation of two different strains in both countries indicates that different strains of the virus may be transferred to each other's ecosystems.

There are limited studies on the molecular characterization of CDV in Turkey. The detected field CD viruses were found to be among the European strains (Ozkul et al. 2004; Oğuzoğlu et al. 2018; Yılmaz et al. 2021). Sequences obtained in the phylogenetic analysis generated with the H gene sequence, were observed to be localized to a branch close to the Italian ancestry, including wild CDV strains under the Arctic CDV lineage (Koç et al. 2021). As a result of the studies, the presence of European and Arctic strains in Turkey has been determined separately until now. However, it has been demonstrated for the first time in our study that strains belonging to two different lineages circulate in the same region.

As a result of the comparison of Arctic strains obtained in the study with other vaccine strains at amino acid level, it was determined that at some points (18., 19., 133., 163., 165. amino acid levels) they were the same in themselves but differed with the vaccine strains (Fig. 3). It comes to mind that these amino acid changes may be a different strain in Arctic like strains, may change the effect of the strain in the vaccine, and may be important in the selection of the vaccine.

Definitive diagnosis of CDV is made by histopathological findings and detection of CDV (RNA) by RT-PCR and antigen by immunohistochemistry. The characteristic histopathological findings of distemper are intranuclear/intracytoplasmic inclusions in the epithelial tissue (gastrointestinal tract, lung, urinary system), brain and less lenfoid tissue. Histopathological examination revealed signs of interstitial

pneumonia in the lungs in all cases, and broncho interstitial pneumonia in three of them. In one case, syncytial cells were noted in the lumen of the alveoli. Intracytoplasmic and intranuclear inclusions were detected in bronchial epithelial cells and syncytial cells. These findings in the lungs are consistent with previous studies in different animal species, including dogs, where the presence of CDV is observed (Pratakpiriya 2017; Michelazzo et al. 2021; Wang et al, 2021b).

CDV usually enters the body by inhalation and it is firstly transported to the oropharyngeal lymph nodes. CDV primarily replicates in immune cells. The virus spreads to the skin, respiratory, gastrointestinal, urinary system epithelial cells and brain. Membrane fusion is necessary for the spread of morbillivirus from cell to cell. Membrane fusion results in the formation of a multinucleated cell known as the syncytium, which is an important cytopathogenic feature of the virus (Rendon Marin et al., 2019). Two epithelial receptors are effective in the spread and aerosol transport of the CDV virus. The first of these is signaling lymphocyte activation molecule (SLAM), which is expressed on B and T lymphocytes, dendritic cells, hematopoietic cells, and macrophages. SLAM plays a key role in the affinity of the virus to distinct species, cell fusion, tropism, virulence and in vitro cytopathogenicity. Mutations in amino acids of the SLAM protein affect the form of infection in distinct species. In studies conducted in distinct species, it has been shown that mutations in amino acids 74 and 129 of SLAM in cells expressing SLAM may contribute to viral infection (Wang et al., 2021a). In addition, in this study, it was determined that the changes in the amino acids of the SLAM protein had an effect on the formation and amount of syncytium. In a study conducted during the CDV epidemic in Switzerland in 2009, it has been determined that functional and structural differences in the H protein of CDV strains are responsible for the change in binding efficiency to SLAM, and as a result, it causes an increase in virus pathogenicity in a wide host spectrum, high morbidity and mortality (Origgi et al. 2012). A second epithelial receptor, Nectin cell adhesion molecule- 4 (nectin-4), a component of adherens junctions of epithelial cells. In various studies on the formation of syncytia, it has been reported that Slam and Nectin-4 receptors, which allow the virus to enter the cell and cell to cell spread, are effective in the formation of syncytia (Rendon Marin et al. 2019; Shin et al. 2022). It has also been reported that morbilliviruses inhibit the JAK/STAT signaling pathway, affecting the natural immunity, and being particularly important in the entry of the virus into the respiratory tract epithelial cells, spreading from cell to cell, and syncytia formation (Shin et al. 2022). Characteristic eosinophilic inclusions frequently cytoplasmic but occasionally intranuclear in the epithelial tissue, intracytoplasmic less frequently the CNS (Maxie, 2015). In present study, multiple syncytia formations, which were in the necrotic follicular the centers, were observed in the spleen of the case with syncytia formation in the lung. Both intracytoplasmic and intranuclear eosinophilic inclusions were also detected in spleen, stomach, pancreas and intestinal epithelial cells. Immunohistochemical analysis revealed CDV antigen was observed in lung, stomach, intestines, pancreas, spleen and brain. CDV antigens were detected more prominently in the demyelinated areas of the brain, bronch/bronchiolar epithelial cells, and syncytial cells.

When the findings obtained as a result of our study are evaluated; presence of the sample, in which syncytia formation was intensively observed, in the Europe strain, unlike the others, raises the question of a relationship between CDV strain and its pathogenesis.

Modified live vaccines, which have been used since the 1950s to combat CDV infection, have provided a great deal of efficacy. Most CDV vaccines are designed based on America-1 strains isolated between the 1940s and 1960s (Onderstepoort, Snyder Hill, Lederle). In recent years, it has been observed that the infection has increased significantly in wild carnivores as well as domestic and stray dogs. Although an intensive vaccination program is implemented today, adequate immunity cannot be provided. This is explained by the fact that the virus can mutate and escape from the immune system. Outbreaks due to the virus and mutations in the genome of the virus give the virus the ability to evade the immune response induced by current vaccines (Ricci et al. 2021). It has been reported that the reason for the detection of the virus in vaccinated animals may be the antigenic differences between the vaccine strain and the wild type of virus, or the immune deficiency or lack of immunity resulting from the effect of maternal antibodies on the vaccine virus (Yeşilbağ et al. 2007). In addition, it is known that field viruses circulating in many countries originate from vaccine viruses and are genetically differentiated.

During the fight against infection, it is necessary to question the protective immunity level of the vaccine, the compatibility of the vaccine with the vaccination schedule, and the presence of maternal antibodies while establishing the vaccination schedule. Conducting genetic studies of circulating strains provides up-to-date information about the evolution of the virus and is also necessary for updating the vaccines that are going to be used.

Declarations

Availability of data and materials

The corresponding author declares that all information regarding this study is available online for public viewing.

Code availability

Not applicable.

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Authors' contributions:

ZK contributed to molecular analyses, MOT contributed to sequence analysis, FI and MU contributed to immunopathological experiments, ZK and FI contributed to manuscript writing drawing of figures and editing. All authors read and approved the final manuscript.

Ethics declarations

Statement of animal rights

The study was approved by the Animal Ethics Committee at Balıkesir University, Turkey (No: 2019/12-2).

Consent to participate

Not applicable.

Consent for publication

Conflict of interest

The authors declare that they have no conflicts of interest.

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gene of canine distemper virus strains detected from breeding foxes, raccoon dogs and minks in China. *Vet Microbiol* 140:34–42. doi: 10.1016/j.vetmic.2009.07.010

Tables

Table1. Oligonucleotide details used for CDV detection in the study

| Primers | Sequences | Genomic position | Product size | References |
|----------------|----------------------------|------------------|--------------|--|
| CDV-Fus-6F | TGTGTATTCGTCTCAGA | 6270-6287 | 797 bp | Romanutti et al. 2016 |
| CDV-Fus-Rev | TAAGACGTGTGACCAGAGTGCTTTAG | 7042-7067 | | |
| 1F-Zhao2010fwd | TTAGGGCTCAGGTAGTCCA | 7057-7075 | 654 bp | Zhao et al. 2010 Trebbien et al. 2014 |
| 2R-7711rev | TGAGATCAAAGACATGGA | 7694-7711 | | |

Table 2. ID, Tissue type, Genbank accession numbers, Genetic lineages of samples infected with CDV

| No | ID | Tissue | Hemagglutinin gene | Fusion gene | Genetic lineage |
|----|---------------|-----------|--------------------|-------------|-----------------|
| 1 | TR/BAL/CDV/53 | Lung | OK184443 | OK244693 | Arctic |
| 2 | TR/BAL/CDV/54 | Liver | OK184444 | OK244694 | Arctic |
| 3 | TR/BAL/CDV/55 | Intestine | OK184445 | OK244695 | Arctic |
| 4 | TR/BAL/CDV/59 | Brain | OK184446 | OK244696 | Arctic |
| 5 | TR/BAL/CDV/60 | Stomach | OK184447 | OK244692 | Europe |

Figures

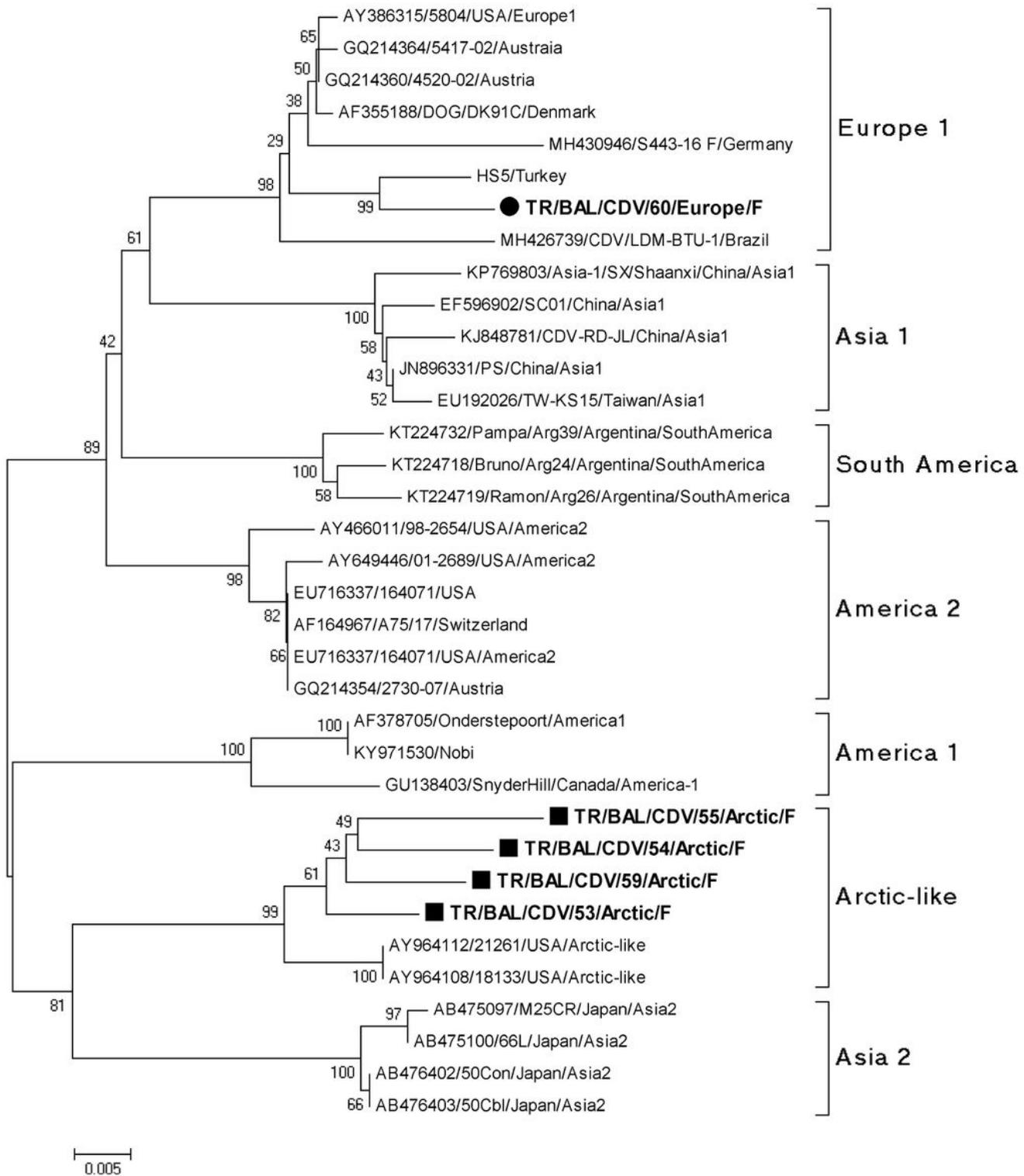


Figure 1

Phylogenetic tree of the nucleotide sequences of the CDV based on partial Fusion gene (Consensus sequences obtained in this study are indicated by solid black square and circle)

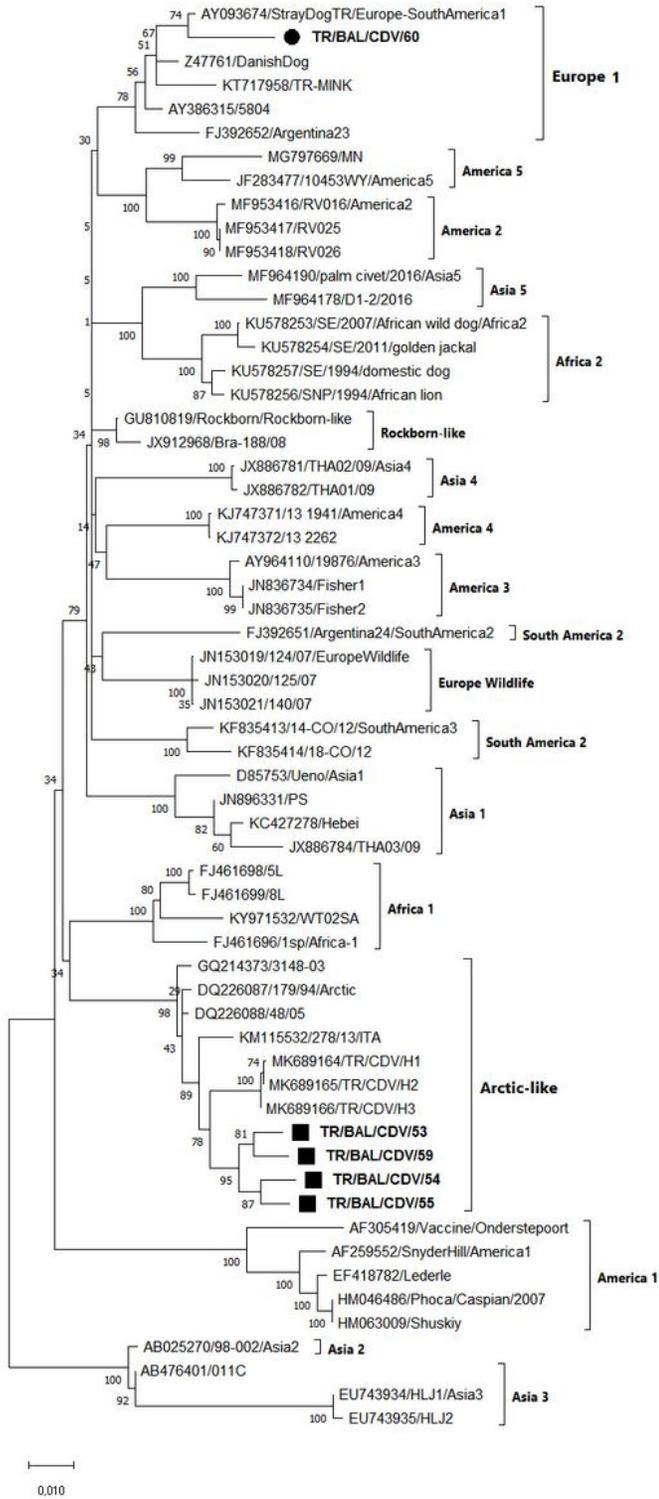


Figure 2

Phylogenetic tree of the nucleotide sequences of the CDV based on partial Hemagglutinin gene (Consensus sequences obtained in this study are indicated by solid black square and circle)

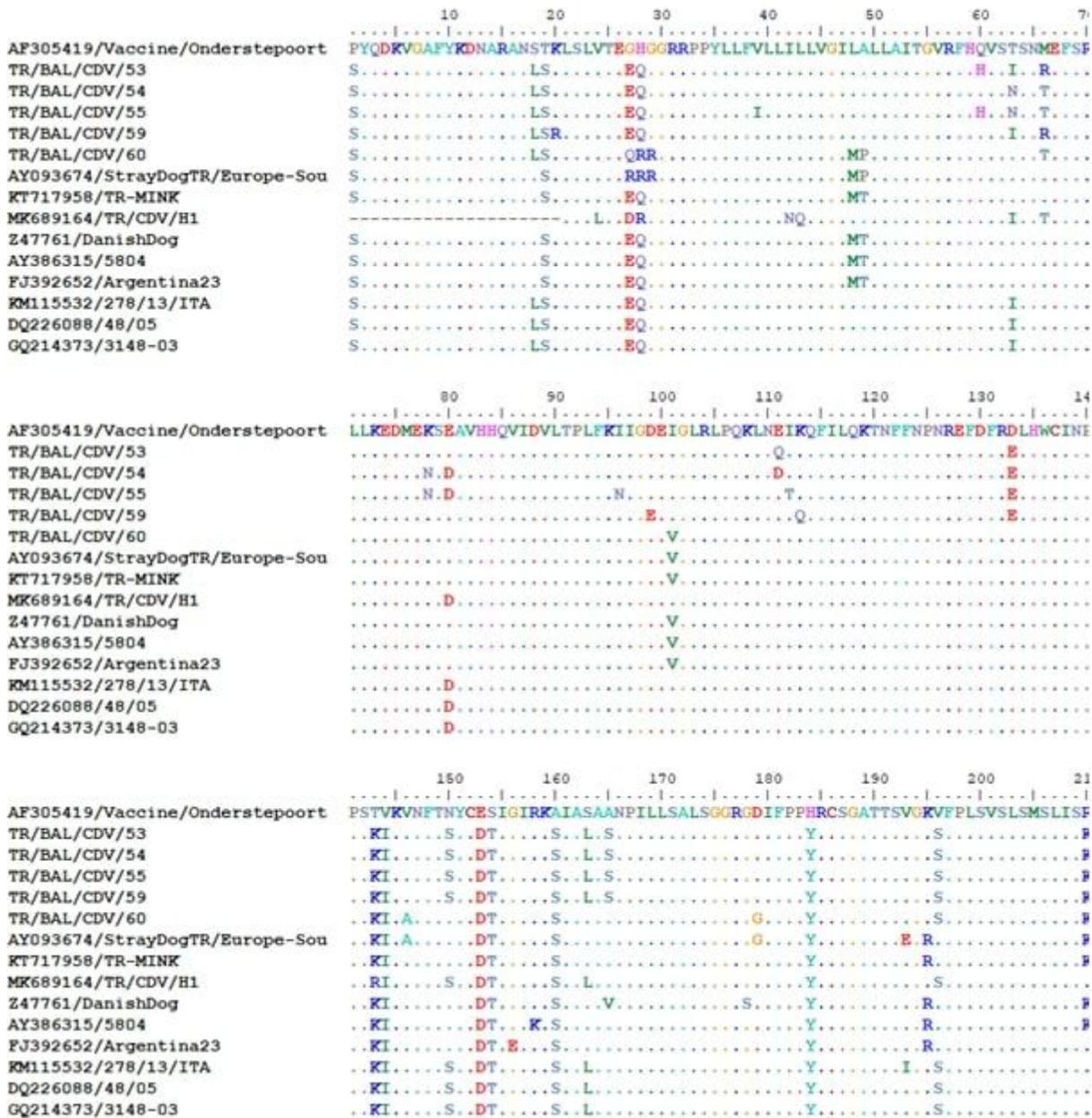


Figure 3

Non-synonymous amino acid substitutions identified in partial H gene sequences obtained in this study.

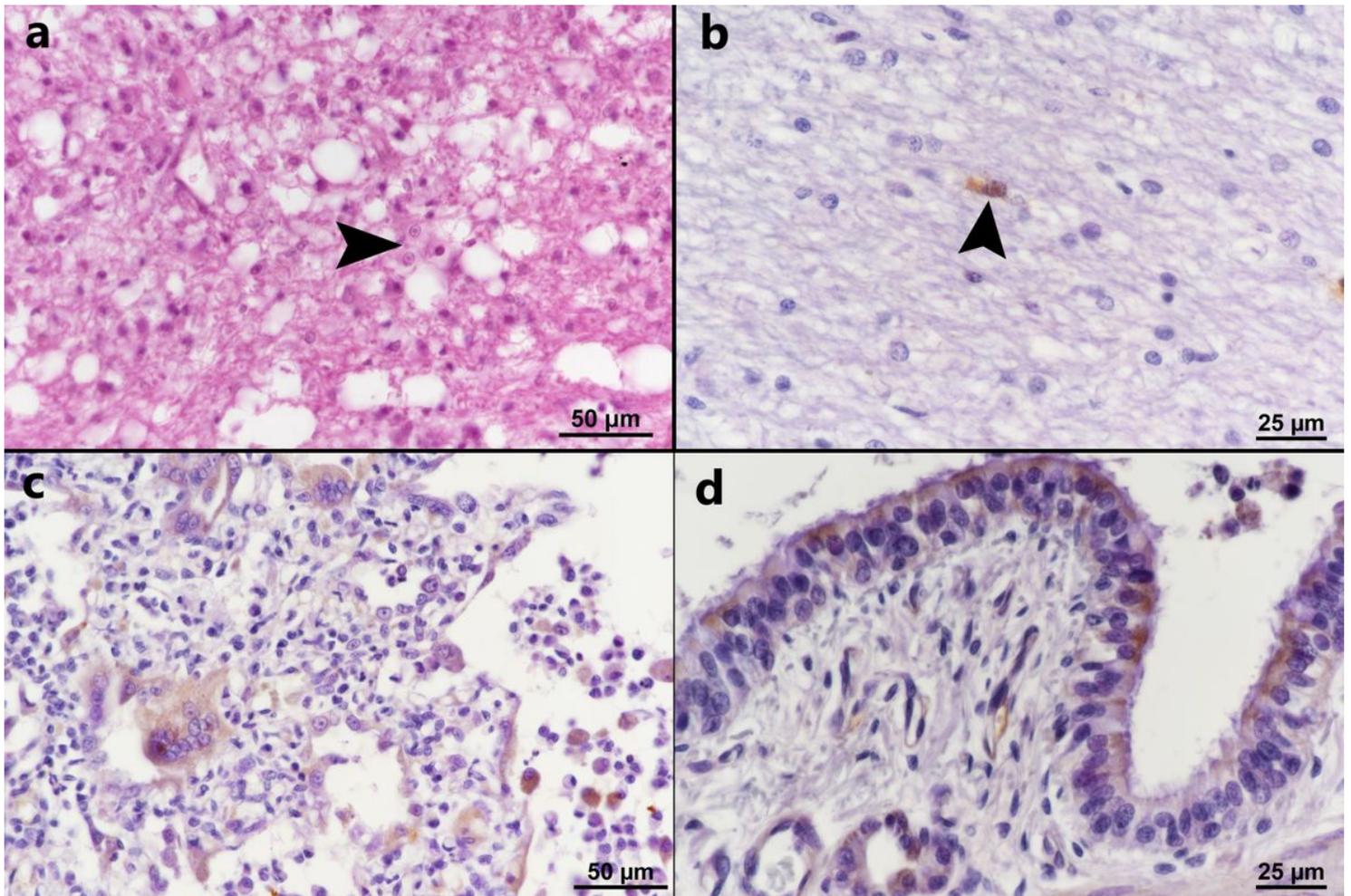


Figure 4

Inclusion bodies (arrowhead), brain, HE (a) Positive immunoreactivity to CDV antigens in the glial cytoplasm and intranuclear inclusion (arrowhead), IHC (b) Syncytial cells with CDV immunopositive inclusions in the lung, IHC (c) CDV antigen positive immunoreactivity in bronchial epithelial cells of lung, IHC (d)

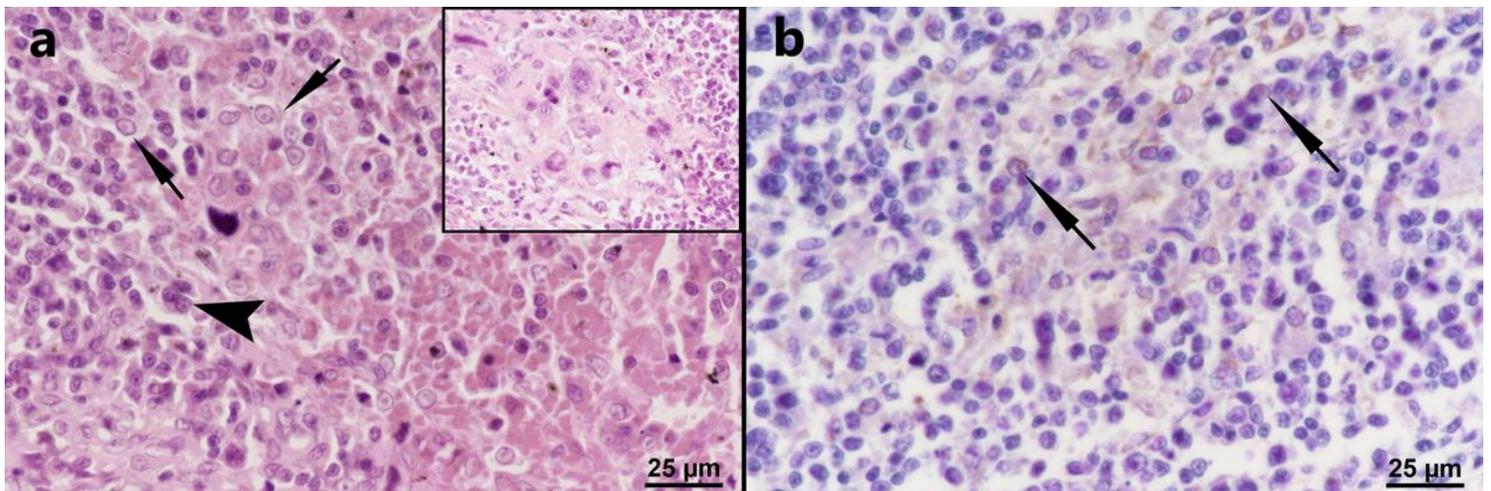


Figure 5

Lymphoid depletion, necrosis in the follicular center, syncytial formation (arrowhead, inset), intracytoplasmic and intranuclear inclusion bodies (arrow) in some of the White pulp of spleen, HE (a). CDV positive immunoreactivity in intracytoplasmic and intranuclear inclusion bodies (arrow) in necrotic areas in the follicles of the spleen. IHC

Figure 6

Intracytoplasmic (arrowhead) and intranuclear (arrow) inclusions in stomach, HE (a). CDV immunopositive intranuclear inclusion in stomach, IHC (b) Positive immunoreactivity to CDV in intranuclear and intracytoplasmic at intestines, IHC (c) Intranuclear inclusions (arrowhead), pancreas, HE (d).