Detection and characterization of Distemper virus in a mink (Neovison vison) in Turkey

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Summary
In this study a mink showing hard pad disease like symptoms was euthanised. Heart blood and various tissue samples collected during necroscopy and tested by specific RT-PCR were found positive for CDV. H and F gene segments of the CDV strain was also partially sequenced using the appropriate primers, and subsequently the sequences were analysed and compared with same gene fragment sequence of other CDV isolates from different countries. The results of the phylogenetic analysis showed that the Turkish-Mink distemper strain is closely related to European CDV strains of lineage 1. Additionally, the distemper antigen was also detected when the tissue samples were examined by histology or immunohistochemistry.

Identificazione e caratterizzazione del virus del Cimurro in un visone (Neovison vison) in Turchia

Riassunto
Nel presente studio sono stati utilizzati campioni prelevati da un visone abbattuto in quanto presentava la "sindrome del piede duro", che è un sintomo clinico caratteristico di infezione da virus del Cimurro (CDV). Tutti i campioni prelevati (sangue intero dal cuore e frammenti di vari organi) sono risultati positivi quando testati mediante una RT-PCR specifica per CDV. Dal ceppo isolato sono stati anche parzialmente sequenziati i geni F e H e le risultanti sequenze sono state allineate e comparate con quelle degli stessi tratti genici di altri CDV provenienti da altri paesi. I risultati dell'analisi filogenetica hanno dimostrato che il ceppo di cimurro rilevato nel visone è strettamente legato ai ceppi europei di lineage 1 del CVD. Il virus è stato inoltre rilevato nelle sezioni di organo sottoposte ad esame istologico e immunoistochimico.

Canine distemper virus (CDV) is an enveloped negative strand RNA virus belonging to *Morbillivirus* genus. This virus infects a broad range of hosts, including wild and domestic carnivores in the *Canidae, Mustelidae, Procyonidae, Felidae, Ursidae, Viverridae, Hyanidae*, and *Pinnipedia* families (Deem *et al.* 2000). The mink (*Neovison vison*) is a member of the *Mustelidae* family. These animals have economic importance because they are frequently farmed for their fur. Other animals from the *Mustelidae* family, including ferrets, have been

models for CDV infection in experimental studies (von Messling *et al.* 2003).

The CDV genome consists of 6 genes, 1 of which, the *hemagglutinin* (H) gene encoding the H protein, is important for viral attachment, the inducing of neutralizing antibodies, and cell tropism (Haas *et al.* 1999, von Messling *et al.* 2001). The H gene has also been used to evaluate genetic diversity among CDV strains, which are genotyped phylogenetically into various lineages (European, American, Asian, African, and Arctic types) based on their H gene sequences

(Nicolin *et al.* 2012, Woma *et al.* 2010). The H protein encoded by the H gene has the largest antigenic variation (Blixenkrone-Moller *et al.* 1992). Another important gene is the *fusion* (F) gene, encoding the F protein. This protein is the most conserved. It works in cytopathogenicity/cell-to-cell fusion and, with the H protein, is the major target antigen for the host immune system (Mochizuki *et al.* 1999).

The main objective of the present study was to describe the distemper infection in a naturally infected mink, and to characterize this distemper strain molecularly according to partial F and H gene sequences. This animal was investigated histopathologically for distemper infection.

To this end, a 1-year-old female mink was sampled from a farm with 11,000 animals, none of which was vaccinated against CDV. The estimated total abortion and mortality rate of 10-15 minks per day was high in this farm. The mink arrived at the Department of Surgery of Veterinary Medicine Faculty, Ankara University (Turkey) in 2011 with an anamnesis of lethargy, fever (41°C), conjunctivitis, sneezing, dyspnoea, oculo-nasal discharge, catarrhal to mucopurulent rhinitis, and tracheitis (Figure 1). Neurological signs were absent. Crusting was seen on the top of the lips and on both sides of the nose (Figure 1). In addition to these lesions, the animal had footpad hyperkeratosis. With the permission of the farm owner, the animal was euthanized under anaesthesia and a necropsy was performed in the Department of Pathology, Medicine Faculty, Ankara University (Turkey). Heart blood and tissue samples from brain, intestine, spleen, liver, lungs, heart, and kidney were collected

and sent to the Department of Virology. All samples were fixed in 10% buffered neutral formalin and routinely processed for paraffin embedding.

RNA was extracted from the obtained whole blood and a mixture of all organs using the Phenol :Chloroform:Isoamylalchohol (25:24:1) technique (Chomcznyski and Sacchi 1987). All obtained samples for diagnostic purpose were tested using reverse transcription-polymerase chain reaction (RT-PCR), with appropriate primers against the F gene (FF1 and FF2) (Barrett *et al.* 1987, Pardo *et al.* 2005). Additionally, the primer pairs FF1/HR2 and HF1/HR1 for F and H genes of the CDV genome were used for sequencing (Lan *et al.* 2005). The amplicons, which were 382 bp, 1301 bp, and 1045 bp, from polymerase chain reaction (PCR) products were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and sequenced using a CEQ 8000 Sequencer (Beckman Coulter, İstanbul, Turkey). Multiple sequence alignments were completed using ClustalW in the Bioedit software program (v.7.2.5) (Hall 1999). The phylogenetic analysis was done for the partial F gene (Figure 2a) and H gene (Figure 2b) sequences using the Mega software program version 6.06 (Tamura *et al.* 2013).

Tissue samples were cut at a nominal tickness of 5 mm, stained with haematoxylin-eosin (HE) and examined microscopically. Immunostaining by the Streptavidine Biotin-Peroxidase Complex (ABC) technique was used to visualize distribution of the viral antigen in tissue sections. For this purpose, the dropped Mouse Anti-Distemper Virus Antibody (CDV-NP, VMRD; diluted 1/125) incubated at 4°C for 18 hours was used as primary antibody. The

Figure 1. *Clinical signs and macroscopical lesions in infected* Neovison vison*, Ankara, Turkey, 2011.* **a** and **b**. Hyperkeratosis on both sides of nose. **c** and **d**. Hyperkeratosis on the footpads. **e**. Enlarged spleen. **f**. Swollen and dark red coloured lung.

3-amino-9-ethylcarbazole (AEC, C01-12, GBI) was used as chromogen while Gill's (I) Hematoxylin as counterstain. The slides were covered with aqueous mounting medium. Tissue sections from a dog with no histopathological evidence of CDV infection were used as negative controls.

Macroscopically, all lobes of the lungs were swollen and dark red colored (Figure 1). Shapeless pale white areas that measured 0.5-2 cm were seen on the lungs. The spleen and liver were also enlarged, and the cut surfaces of both organs were haemorragic.

The lungs were characterised histologically by interstitial pneumonia. Mononuclear-cell infiltration was seen around the bronchus and bronchioles. The alveolar walls were thickened by infiltration of mononuclear cells and pneumocyte proliferations. In the affected areas and near the pleura, the alveoli were lined by cuboidal cells. Additionally, amounts of proteinaceous fluid, a few neutrophils, and desquamated epithelial cells were observed. The most conspicuous histological findings were eosinophilic cytoplasmic and nuclear inclusion bodies.
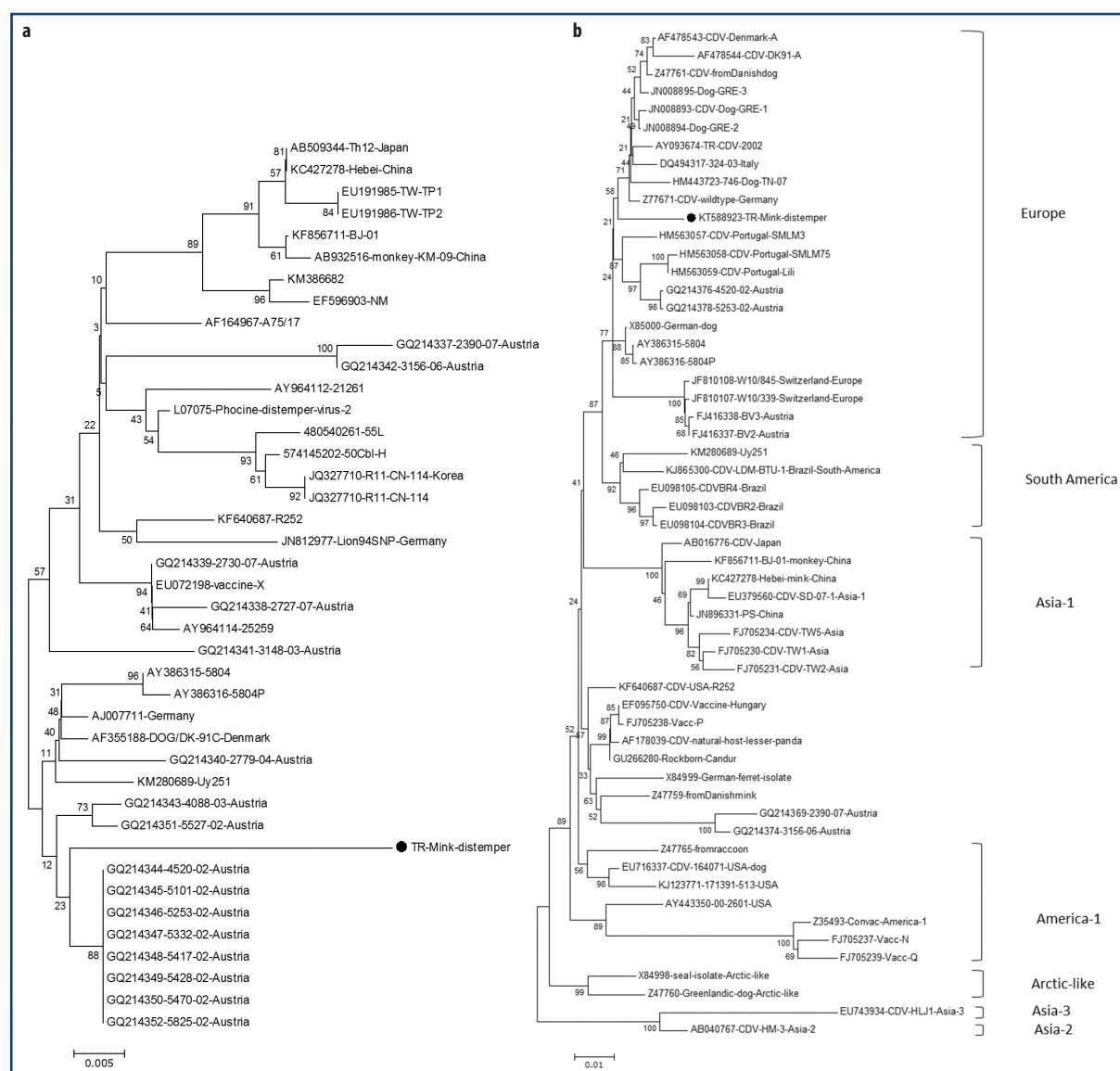

Figure 2. *The phylogenetic relationship of selected strains of canine distemper virus based on the F and H gene partial sequences.* The phylogenetic trees were inferred using the Neighbor-Joining method in MEGA6 software (Tamura *et al.* 2013). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. **a**. Phylogenetic tree based on nucleotide sequences of the F gene of Turkish mink distemper strain (accession number: KT588922) and Canine distemper virus (CDV) strains from other countries. The optimal tree with the sum of branch length = 0.26428231 is shown. The analysis involved 41 nucleotide sequences. There were a total of 378 positions in the final dataset. **b**. Evolutionary analysis of the H gene for Turkish mink distemper strain (accession number: KT588923) was conducted by using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.61347138 is shown. The analysis involved 56 nucleotide sequences. There were a total of 928 positions in the final dataset.

The alveolar septa were expanded by a mononuclear-cell infiltrate. Cytoplasmic and intranuclear inclusion bodies (Figure 4a) were also observed inside the bronchiolar and alveolar cells. Immunohistochemically, the labelling of the viral antigen was observed at the bronchiolar cells, alveolar cells, and macrophages in the lungs (Figure 4b), as well as at the epithelia of the footpads (Figure 4c), the lymphocytes in the spleen (Figure 4d), and the epithelia of the bile duct in the liver.

The samples obtained for diagnostic purposes and tested by specific RT-PCR were found positive for CDV. The determined nucleotide sequences of the F and H gene amplicons were deposited into GenBank.

The accession numbers are KT588922, KT588923, and KT717958 (F gene, position 5505-5886; H gene, position 7962-9007; partial F and partial H genes, position 6897-8198). Phylogenetically, the highest homology of the Turkish mink distemper strain F gene was with Austrian wild carnivore samples, which have accession numbers between GQ214344 and GQ214350 (Figure 2a).

The obtained mink distemper H gene sequence could only be compared with the single Turkish dog CDV sequence deposited in GenBank (Figure 2b). It was more closely related to the European CDV strains, based on the H gene sequence analysis. Similarly to the F gene sequence, Turkish mink
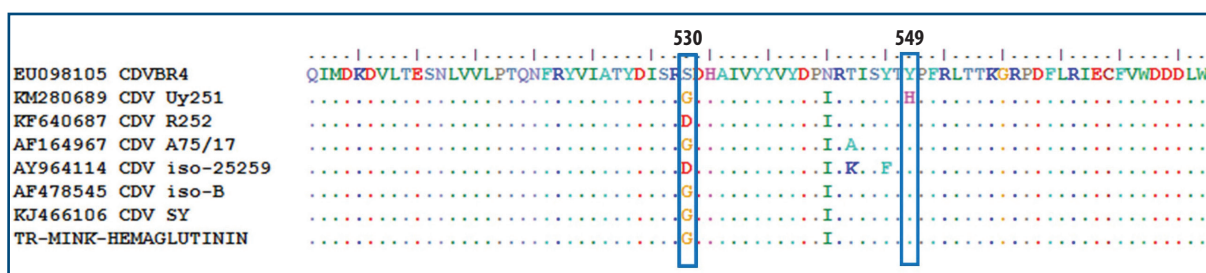

Figure 3. *The comparison of 530. and. 549.aa substitutions of hemagglutinin gene of Canine distemper virus.*
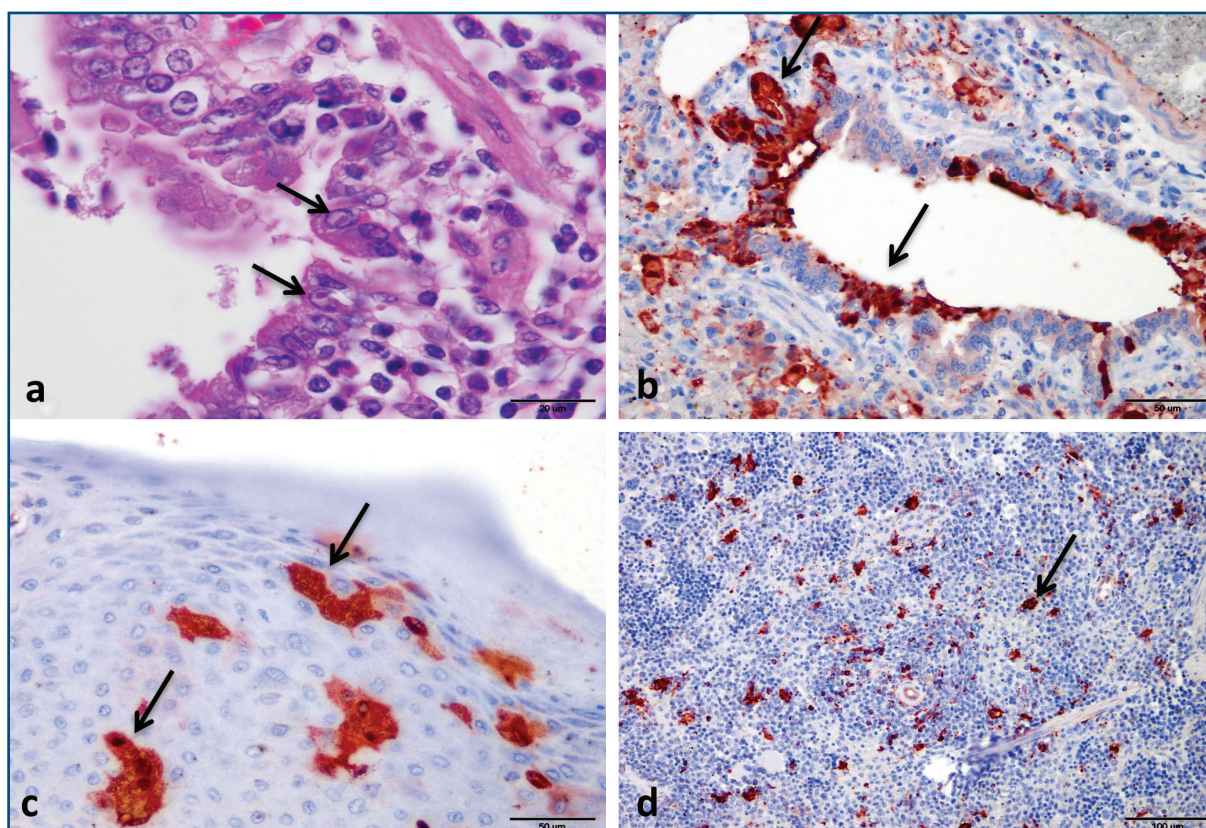

Figure 4. a. *Lung. Acidophilic intranuclear inclusion bodies in bronchial epithelial cells of mink lung (arrows). Mononuclear cell infiltration around the bronchiole. HE. Bar = 20 μm.* **b**. *Lung. Canine distemper virus antigen in the alveolar epithelial cells, bronchial epithelial cells (arrows) and macrophages. Immunohistochemistry. Bar = 50 μm.* **c**. *Footpad. Canine distemper virus antigen in the epithelial cells (arrows). Immunohistochemistry. Bar = 50 μm.* **d**. *Spleen. Canine distemper virus antigen in the lymphocytes (arrows). Immunohistochemistry. Bar = 100 μm.*

distemper strain H gene sequence is more closely related to the Austrian wild carnivore strains than to the Turkish dog CDV strain (Figure 2a). Presence of aa substitutions at the 530 (glycine, G) and 549 (tyrosine, Y) positions were also found in the H gene of the Turkish mink distemper strain (Figure 3).

In Turkey, CDV infections have been reported several times in domestic dogs (Börkü *et al.* 2012, Çalışkan and Burgu 2007, Ozkul *et al.* 2004, Toplu and Yazicioglu 2008). However, to the best of our knowledge, is the first time that clinical signs due to CDV infection were described in captive or free-ranging Mustelids in this country.

Based on information from the farmer, this was the fourth case on the farm in 25 years.

The infected animal showed classical symptom for CDV (hard pad disease) (Figure 1), which coincided with the histopathological results of CDV infection. It is known that hard pad disease is a dermatological sign of CDV infection and is characterised by hyperkeratosis, a familiar pathognomonic sign of the disease (Caswell and Williams 2007). We found the CDV antigen on the footpads of the animal (Figure 4c). Although inclusion bodies were not histopathologically identified within the epithelia of the footpads, lymphocytes in spleen and epithelium of the bile duct in the liver, the CDV antigen was observed within these cells immunohistochemically.

Macroscopic lesions were similar to those in other studies. For example, lungs with foci of consolidation, as well as enlarged spleens and livers, have been reported in foxes (López-Peña *et al.* 1994), ferrets (Perpiñán *et al.* 2008), and minks (Keymer and Epps 1969).

Neurological signs are commonly seen in animals with CDV (Zhao *et al.* 2015). However, no neurological signs have been reported in our study, neither clinically nor pathologically. Also, microscopic lesions of the mink respiratory system closely resembled those of CDV infected dogs (Caswell and Williams 2007). The antigen was detected immunohistochemically within the bronchiolar cells, alveolar cells, and macrophages in lungs. In addition, cytoplasmic and intranuclear inclusion bodies were observed inside the bronchiolar and alveolar cells (Figure 4a-b). Therefore, the pathological findings in the respiratory system were consistent with active CDV infection.

Based on phylogenetic analyses of H gene, CDV strains cluster at 10 lineages, namely America-1, America-2, South America, Asia-1, Asia-2, Asia-3, Southern Africa, Arctic-like, Europe, and Europe wildlife (Nicolin *et al.* 2012, Renteria-Solis *et al.* 2014, Trebbien *et al.* 2014, Zhao *et al.* 2010). The phylogenetic analysis based on partial F (Figure 2a) and H (Figure 2b) protein coding region sequences revealed that the virus detected in this case is genetically related to viruses of European lineage. Furthermore, given the F gene sequences, it is possible to conclude that the virus was more closely related to wild carnivore distemper viruses.

The mink breeder mentioned that unclaimed stray dogs were roaming near the mink farm. The farm was surrounded by wire fence and may also be open to wild carnivore infections. In farms separated with wire fence and not respecting the biosafety rules, transmission routes of the diseases must be taken into consideration.

McCarthy and colleagues (McCarthy *et al.* 2007) reported that, by means of some amino acid (aa) changes at SLAM (signaling lymphocytic activation molecule), the receptor-binding sites 530 aa and 549 aa in the H protein identify the host specificity of CDV strains. In the present study, the mentioned aa differences of the H gene have been confirmed for the Turkish mink distemper strain. The presence of aa substitutions at the 530 (*glycine*, G) and 549 (*tyrosine*, Y) positions in the H gene of the Turkish mink distemper strain may stand for a dog origin of this strain (Figure 3). Had the gene not been from a dog host origin the substitutions in 530 aa and 549 aa were expected to be R/D/N and H, respectively. Additionally, the same aa substitutions were reported for Turkish Dog Distemper Virus strain in 2002 (530 G, 549 Y) (Nicolin *et al.* 2012). In our opinion, the findings of this study support the farmer's theory about stray dog transmission.

It is known that Mustelids should be immunised routinely against CDV infection (Fox 1998, Brown and Purcell 1999). In Turkey, no distemper vaccine is available for mink, and the vaccines are only authorised for domestic dogs. At the same time, the present vaccines against CDV infection provide an immune response regardless of the strains from the different lineages (Lan *et al.*, 2006). In total, approximately 3000 animals died during the CDV outbreak on this farm. The mortality rate was estimated at 27.27% (3,000/11,000) based on the farmer's declaration. Therefore, we recommend the vaccination, which could be of vital importance for the prevention of the CDV infection in minks.

In conclusion, since the first description in 1905 by Carré in France of the CDV infection in minks (van Rooyen 1949), the disease has been researched worldwide. The results of our phylogenetic analysis of the F and H genes of CDV genome show that the Turkish mink distemper strain in this study was genetically related to CDVs previously detected throughout the world. Although the substitutions of SLAM receptor binding sites showed that the spread of CDV infection was from dogs to non-dog hosts and vice-versa, it should not be forgotten that wild carnivores could be a source of distemper infection for domestic carnivores.

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