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Canine Distemper Virus in a Wild Far Eastern Leopard (*Panthera pardus orientalis*)

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ABSTRACT: The critically endangered population of Far Eastern leopards (*Panthera pardus orientalis*) may number as few as 60 individuals and is at risk from stochastic processes such as infectious disease. During May 2015, a case of canine distemper virus (CDV) was diagnosed in a wild leopard exhibiting severe neurologic disease in the Russian territory of Primorskii Krai. Amplified sequences of the CDV hemagglutinin gene and phosphoprotein gene aligned within the Arctic-like clade of CDV, which includes viruses from elsewhere in Russia, China, Europe, and North America. Histologic examination of cerebral tissue revealed perivascular lymphoid cuffing and demyelination of the white matter consistent with CDV infection. Neutralizing antibodies against CDV were detected in archived serum from two wild Far Eastern leopards sampled during 1993–94, confirming previous exposure in the population. This leopard population is likely too small to maintain circulation of CDV, suggesting that infections arise from spillover from more-abundant domestic or wild carnivore reservoirs. Increasing the population size and establishment of additional populations of leopards would be important steps toward securing the future of this subspecies and reducing the risk posed by future outbreaks of CDV or other infectious diseases.

Key words: Amur leopard, canine distemper virus, Far Eastern leopard, *Panthera pardus orientalis*.

The Far Eastern leopard (FEL; *Panthera pardus orientalis*), also known as the Amur leopard, is one of the most-threatened large felids and is currently classified as Critically Endangered by the International Union for Conservation of Nature (Stein et al. 2016). Populations formerly extended across northeast China, the Korean Peninsula, and southern Primorskii Krai (province) in the Russian Far East (Hebblewhite et al. 2011). Habitat modification, prey depletion, and poaching have reduced the FEL to a single subpopulation occupying approximately 7,000 km^2 in southwestern Primorskii Krai and neighboring Jilin province in China (Hebblewhite et al. 2011). With approximately fewer than 60 individuals remaining (Vitkalova and Shevtsova 2016), this genetically impoverished population is at risk from stochastic processes including inbreeding depression, environmental calamities, and disease (Uphyrkina et al. 2002).

On 8 May 2015, a female leopard approximately 2 yr old was found close to a road that runs through the Land of the Leopard National Park in Khasanskii district (42°57′19.4″N, 131°21′5.4″E). On initial approach, the leopard showed a lack of fear toward people and vehicles and an indifference to its surroundings. However, it was able to respond to movement, suggesting a continuity of vision. The leopard was immobilized

using 5 mg/kg tiletamine and zolazepam (Zoletil, Virbac, Carros, Provence, France) and was taken to the Alekseevka PRNCO "Tiger Center." The animal weighed 28 kg upon admission and was considered to be in moderately poor body condition. During care, the leopard was uninterested in food and water and required hand-feeding and intravenous fluid therapy. Intramuscular injections of 5 mg/kg of enrofloxacin (Baytril Bayer, Leverkusen, Germany) and 0.2 mg/kg dexamethasone (Dalkhimfarm, Khabarovsk, Russia) were given on a weekly basis.

We collected blood samples on 8 and 16 May 2015 using ethylenediaminetetraacetic acid as an anticoagulant and submitted them for analysis at the Alba Laboratory, Vladivostok. Manual counts of white blood cells were within normal ranges for domestic cats (7.3×10^9/L on 8 May and 10.3×10^9/L on 16 May). All other cytology and biochemistry values also fell within the ranges considered normal for domestic cats by the laboratory.

Despite supportive care and medical treatment, the leopard's movements became increasingly uncoordinated, with progressively severe hind limb contractions that could not be controlled with intravenous midazolam (Hoffmann-La Roche, Basel, Switzerland, 0.05–0.01 mg/kg). The leopard's condition degenerated and euthanasia was performed on 25 May for ethical reasons.

Postmortem examination was performed on 25 May and samples (brain, spinal cord, lung, trachea, heart, esophagus, stomach, small intestine, large intestine, cecum, pancreas, liver, kidney, bladder, ovary, uterus, spleen, lymph node, adrenal glands, skeletal muscle, skin, and tongue) were fixed in 10% neutral buffered formalin for histologic examination. Additionally, 50 μg each of brain and lung were collected in 1 mL of nucleic acid stabilizer (RNALater, Sigma-Aldrich, St. Louis, Missouri, USA) for analysis using reverse transcriptase PCR. We extracted DNA and RNA with the AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, California, USA) following the manufacturer's instructions. One-step reverse transcriptase PCR amplification of CDV was performed based on the protocols used by Seimon et al. (2013) using pan-morbillivirus primers targeting the phosphoprotein (*P*) gene (Barrett et al. 1993) and CDV-specific primers (4Farctic/4R: 5′-ATCCCTCATGTGTTATCATT-3′ and 5′-GACCTCAGGGTATAGAATCTGG-3′, adapted from Müller et al. 2011) targeting the hemagglutinin (*H*) gene. We purified the PCR products of correct molecular weight using the ExoSAP-IT reagent (Affymetrix, Santa Clara, California, USA) and directly sequenced in the forward and reverse directions using a GA3130 DNA Analyzer (Applied Biosystems, Foster City, California, USA). A 389-base pair trimmed fragment of the phosphoprotein (*P*) gene (KY807294) and a 528-base pair trimmed fragment of the *H* gene (KY807293) were amplified from brain tissue only. Based on Blastn analysis (National Center for Biotechnology Information, Bethesda, Maryland, USA), the *H* gene was 97% identical to a sequence from an Italian dog in 2013 (GenBank accession no. KF914669), and the *P* gene shared 99% identity with a sequence from an Amur tiger (*Panthera tigris altaica*) in the neighboring territory of Khabarovskii Krai in 2003 (KC579361).

To show the relationship between the leopard CDV strain and those from other regions, a phylogenetic tree was constructed for the *H* gene using Bayesian inference (Fig. 1). The appropriate nucleotide substitution model general time reversible was selected based on Akaike Information Criterion scores using jModeltest 2 version 2.1.8 (Darriba et al. 2012). The tree was constructed using the Geneious MrBayes plug-in (version 3.2.6, Biomatters Ltd., Auckland, New Zealand) with 1,100,000 iterations, subsampling every 200 trees, and discarding 25% of samples as burn-in (Huelsenbeck and Ronquist 2001). Consistent topology was found in a consensus tree constructed using maximum likelihood analysis. Genetic and antigenic drift has driven the divergence of CDV into distinct lineages, which follow a broadly geographic pattern (Harder et al. 1996; Haas et al. 1997; Martella et al. 2002). The *H* gene amplicon obtained from the leopard fell within the Arctic-like clade of CDV (Haas et al. 1997;

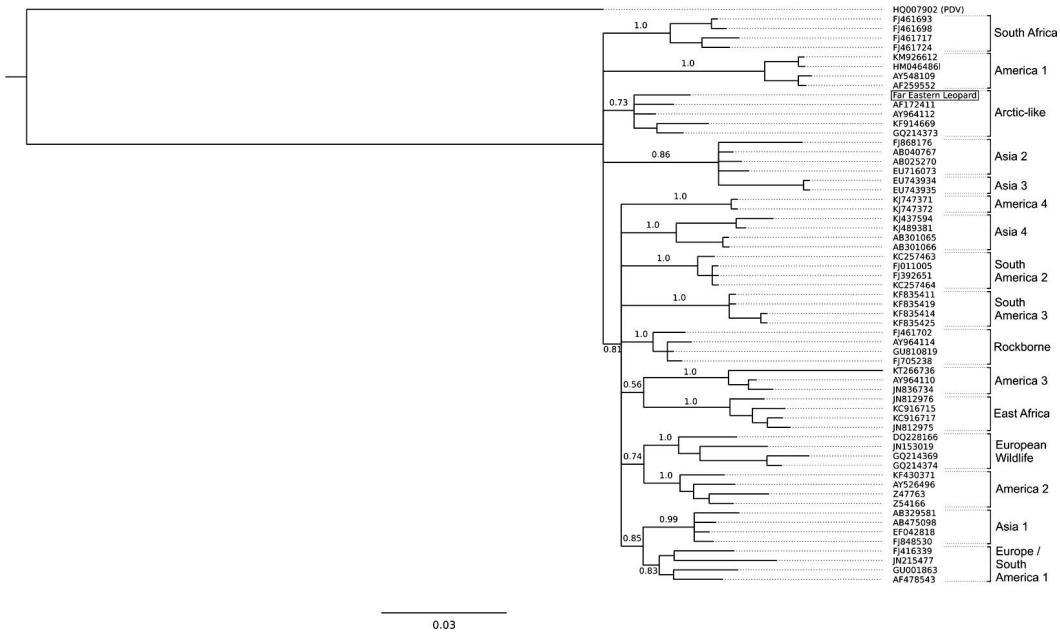

FIGURE 1. A phylogenetic tree illustrating the relationship of a canine distemper virus sequence obtained from a wild Far Eastern leopard (*Panthera pardus orientalis*) that was euthanized due to advanced neurologic disease. The tree was constructed using the hemagglutinin (*H*) gene obtained from Far Eastern leopard (within box) with 59 previously published *H* genes. Noncoding nucleotides were excluded, resulting in an alignment of 382 base pairs in length. Analyses were performed using MrBayes (Bayesian inference) and the general time reversible substitution model with 1,100,000 iterations, subsampling every 200 trees, and discarding 25% as burn-in. A phocine distemper virus (PDV) sequence was included as an outgroup (GenBank accession no. HQ007902).

Fig. 1), which includes sequences obtained from Amur tigers in the Russian Far East and from Baikal seals (*Phoca sibirica*) from Lake Baikal (Seimon et al. 2013).

Multiple, replicate, 5-µm sections of the brain (cerebrum) and lung were stained with hematoxylin and eosin and examined locally by A. Markina (White Fang Veterinary Clinic, Moscow, Russia), and were scanned (using a Panoramic MIDI II scanner, 3DHistech Ltd., Budapest, Hungary) and examined remotely by D. McAloose using CaseViewer software (3DHistech Ltd.). Mild to moderate, multifocal, nonsuppurative, lymphoplasmacytic meningeal and perivascular inflammation was present in the brain. The inflammation was associated with multifocal areas of mild to moderate demyelination and vacuolization of the white matter and mild multifocal gliosis. Viral inclusions typical of infection were suspected but not confirmed (image capture/resolution and cell detail were less than optimal at high [400× and 630×] magnification). The pattern and type of inflammation, in particular the perivascular lymphoid cuffing along with demyelination of the white matter, were highly suggestive of CDV infection despite the lack of identifiable inclusions. No notable histologic lesions were identified in sections of lung, lymph node, or kidney.

The leopard was diagnosed as being infected with CDV based on the detection of *H* gene and *P* gene sequences, the pattern and type of inflammation, consistent demyelination in the brain, and clinical signs consistent with an advanced neurologic disease associated with CDV infection. This is the first case of CDV recorded in a wild leopard, although infections have previously been reported in captive leopards in the US (including one Chinese leopard [*Panthera pardus japonesis*] and two of unspecified subspecies; Appel et al. 1994), and antibodies to the virus have been reported in a single, free-ranging African

leopard (*Panthera pardus pardus*) in Kenya (Kock et al. 1998). To assess the history of exposure of the wild FEL population, we submitted archived serum samples collected from 10 individuals between 1993 and 2008 to the Washington State Disease Diagnostics Laboratory (Pullman, Washington, USA) for analysis using virus neutralization. Neutralizing antibodies to the Onderstepoort strain of CDV were measured using the protocols of Appel and Robson (1973). Antibodies were detected in two female FELs, estimated to be 3 and 4 yr old and sampled during June 1993 and August 1994 (with titers of 1:64 and 1:256, respectively), confirming previous exposure in the population. Both of these animals survived for at least 14 and 38 mo after sampling, respectively, based on radiotelemetry data.

Dogs infected with CDV typically manifest with an upper respiratory disease, with or without systemic signs of pyrexia, dermatitis, hyperkeratosis, or enteritis (Greene and Appel 2006). Neurologic signs often follow but are not present in all cases, being found more commonly with certain viral strains (Summers et al. 1984). The wild leopard presented to us only with neurologic signs; there was no respiratory or systemic disease. Likewise, lesions consistent with CDV infection were only found in brain tissue (although immunohistochemistry and in situ hybridization, which might have demonstrated virus in other tissues, were not available). Viral amplicons were detected in brain but not in lung tissue. We do not know if respiratory or systemic signs might have been present during an earlier stage of infection or whether disease was restricted to neurologic tissue. Behavioral abnormalities may allow for the detection of neurologic cases in free-ranging felids, leading to under-recording of systemic disease. However, infection of epithelial tissue during systemic disease is crucial to CDV transmission (Sawatsky et al. 2012); therefore, the pathogenesis of CDV in wild felids has important epidemiologic implications and warrants investigation.

The exposure of wild FELs and the mortality of at least one individual is an important finding, given the sensitivity of small populations to stochastic events. Populations of threatened carnivores are typically too small to maintain pathogens such as CDV, which cause short-lasting infections, necessitating spillover from more-abundant reservoir hosts (Woodroffe 1999). In Primorskii Krai, potential reservoir populations included domestic dogs and abundant wild mesocarnivores such as sable (*Martes zibellina*), raccoon dogs (*Nyctereutes procyonoides*), and Asian badgers (*Meles leucurus*). Infections of CDV have also been recorded among Amur tigers in Russia, including at least one tiger close to the restricted range of FELs (Quigley et al. 2010; Seimon et al. 2013). The threat of CDV adds to the case for establishing additional wild populations of leopards as insurance to buffer against future outbreaks or the creation of habitat corridors to promote colonization of new areas through natural dispersal.

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