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Case report

Molecular diagnosis with the corresponding clinical symptoms of canine distemper virus infection in javan leopard (*Panthera pardus* ssp. *melas*)

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ABSTRACT

Diseases are one of the possible threats to the conservation of wild cat populations. One disease that has been reported to infect and cause death, including in various wildlife, is the canine distemper virus (CDV). Here, we report the first case of CDV in an adolescent melanistic Javan female leopard in Indonesia. We combined the clinical report with the Reverse Transcription PCR (RT-PCR) analyses on the faecal and brain samples of partial nucleoprotein (*CDV-N*) and hemagglutinin (*CDV-H*) genes. We also performed analyses of urine, haematology, and blood chemistry. The *CDV-H* nucleotide sequence confirmed the CDV infection in the female leopard and was clustered to the CDV's Asia 1 genotype. This finding opens an investigating window to analyse the pathogen transmission between animals in wildlife, particularly to support the management of conservation in natural habitats in Indonesia.

1. Introduction

Canine distemper virus (CDV) infections cause a disease known as canine distemper which can affect carnivores around the world. The virus is highly contagious and spreads through contact with fluids and aerosol droplets [1]. CDV is considered the most contagious among morbilliviruses [2] and leads to an epizootic of high mortality [3].

First detected in 1760 [4,5], CDV can be transmitted to other carnivore hosts including Canidae, Procyonidae, Mustelidae, Ursidae, Ailuridae and Felidae, and different orders, Artiodactyla, Primates, Rodentia, and Proboscidea, posing a possible decrease in wild [6] and captive populations [7, 8]. This emerging disease has been confirmed to infect large wild Felids, such as Amur and Sumatran tigers [9, 10], African and Asiatic lions [11, 12], and Far Eastern and Indian leopards [12, 13].

Individuals infected with CDV will generally develop severe respiratory conditions, such as immunosuppression, gastrointestinal complications, and/or neurological deficiencies and conjunctivitis [2]. As a neurologic disease with encephalitis, there is no record of an infected animal that survived CDV [6]. Here, we present the first case of a CDV infection in a wild Javan leopard (*Panthera pardus* ssp. *melas*) in Indonesia and genetic analysis of the virus with other known global CDV strains and cases.

2. Case report

An adolescent wild female melanistic Javan leopard (later named Rita) was seen by tourists on the 15th of December 2020 in Carita Grand Forest Park, Banten, showing benign and debilitating behaviour. The leopard was rescued by the West Java Nature Conservation Agency (Balai

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Konservasi Sumber Daya Alam – BKSDA Jawa Barat) and treated and observed by the Animal Sanctuary Trust Indonesia (Yayasan Alam Satwa Tatar Indonesia – ASTI) between 16th and 27th of December 2020, but failed to survive.

Upon arrival at the ASTI quarantine facility, Rita was described as a sub-adult female weighing 15–20 kg, which fits into category 2 of the Body Condition Score (BCS) [14]. Whilst still somewhat mobile and alert, Rita appeared lethargic. She was calm, did not seem threatened when approached by humans, and even tried to approach the staff who observed her from outside the cage. On the 17th of December 2020, Rita received 1 ml/5 kg of adenosine triphosphate (ATP) (BIOSAN®, PT. Sanbe Farma, Indonesia) and hematopoietic (HEMATODIN®, PT Sanbe Farma, Indonesia) injections, multivitamins (INJECTAMIN®, PT Sanbe Farma, Indonesia), and a long-acting 15 mg/kg of Amoxicillin (Intramox-LA®, Interchemie, Holland) as part of a standard antibiotic treatment regime. She experienced seizures on a couple of occasions between the 16th-18th of December 2020 and discharge was observed from both eyes. Her left eye was closed most of the time, however the nictitating membrane was red, and the discharge was greenish. On the 18th of December 2020, a faecal sample was collected and tested using Reverse Transcription PCR for the CDV nucleoprotein (N) gene [referred to 15], which showed a positive result with a 419 bp amplicon. Her breathing appeared heavy and nasal flarings were also observed. Raw chicken and beef, boiled chicken meat, and wet cat food were offered during these dates, however Rita refused to eat. She also refused drinking the multivitamin mixed water offered.

From the 19th to the 21st of December 2020, Rita showed health improvements. Starting from the 19th of December 2020, she was given lactated Ringer's infusion subcutaneously, 1 mg/kg of Bromhexine (mucolytic), 0.5 mg/kg of anti-inflammation Dexamethasone (Glucortin®, Interchemie, Holland) orally, 1 ml/5 kg of adenosine triphosphate (ATP; BIOSAN®, PT Sanbe Farma, Indonesia), hematopoietic (HEMATODIN®, PT Sanbe Farma, Indonesia), and multivitamin injections (INJECTAMIN®, PT Sanbe Farma, Indonesia). 5 mg/kg of Enrofloxacin (ROXINE®, PT Sanbe Farma, Indonesia) was injected to replace the Amoxicillin mentioned above. She started to eat soft-liquid food three times a day and groomed herself after eating. She appeared more alert and aggressive, hissing and growling when she was approached or when she appeared annoyed. However, her breathing was still laboured, and nasal flarings continued. Rita was observed to stop breathing for 5 s at 19.00 on the 21st of December 2020, and her condition subsequently deteriorated. Rita progressed to experience seizures a couple of times a day. Her footpads were thick (hyperkeratosis), a common sign of canine distemper disease. She refused to eat and drink leading to noticeable weight loss.

On the 23rd of December 2020, Rita experienced a series of seizures at dusk and was given 0.25 mg/kg of Acepromazine (Castran®, Interchemie, Holland). Her basic profile haematology test showed a higher mean corpuscular haemoglobin concentration (MCHC) and low lymphocyte level (lymphopenia). Her blood chemistry test result showed low blood urea nitrogen (BUN), total protein (TP), globulin, and alkaline phosphatase (ALP) levels. She also had a pale pink mucosa with a capillary refill time (CRT) of less than 3 s. Later that evening, Rita again experienced a series of seizures with 15–30 s duration every 10 min until the following day. 0.25 mg/kg of Diazepam (PT Meprofarm, Indonesia) was finally obtained and administered in the late afternoon and repeated continually every 12 h. Diazepam significantly reduced Rita's seizures, with an occasional relapse every 12–15 h. Thorax auscultation revealed that there were rare sounds in all lung fields and Rita was found dead at 04.45 (approximate time of death estimated at 04.00) on the 27th of December 2020. A post-mortem physical examination determined that Rita's body condition score was abysmal, with a score of 1 (scale of 1–5) [14], and her body weight was 10.5 kg (Figure 1 A-F).

In order to avoid animal suffering and to raise animal welfare, the present study followed international guidelines for humane animal treatment and complied with legislation from the World Organisation for Animal Health (OIE)'s animal welfare standards [16], World Zoo and Aquarium Animal Welfare Strategy [17], and International Union for Conservation of Nature (IUCN) guidelines for the management of confiscated, live organisms [18].

3. Materials and methods

3.1. Ethics approval

Ethical approval was not sought because the present study is not an experimental animal study. Instead, the study focuses on the diagnosis of confiscated animals in the rescue center. Nonetheless, this study has transport and study approval by the Ministry of Environment and Forestry, Indonesia (No. SI.103/K.1/BKW1/KSA/12/2021) as the biological source of laboratory analysis in this study.

3.2. Specimen collection

Rita's death raised concerns of possible CDV infections within wild Javan leopards. ASTI sent Rita's faecal samples to the Research Centre, Bogor Agricultural University (IPB University), to obtain a more detailed examination of this case. This sample was sent for the first molecular analysis of suspected CDV (partial nucleoprotein *CDV-N*) on the 17th of December 2020. A year later (10th of December 2021), the carcass arrived at the Research Centre, IPB University, for further analysis. The carcass had been stored in the −20 °C freezer ASTI whilst awaiting a transportation permit.

3.3. Urine, haematology, and blood chemistry test

Urine samples were collected on the 17th of December 2020 using a pipette from the cage floor after Rita was seen urinating for a routine urine test. Blood samples were taken on the 22nd of December 2020 using a butterfly IV catheter through the Saphenous Vein for haematology and blood chemistry tests. Rita's weak physical condition allowed sampling to be carried out without anaesthesia. Both samples were sent the same day they were collected for our laboratory test at Research Centre, IPB University.

3.4. RNA extraction

Faecal and brain samples from the Javan leopard were extracted to obtain viral RNA using the QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany). The RNA is then reverse transcribed to obtain the cDNA. Following the manufacturer's instructions, we used the Superscript™ III First-Strand cDNA Synthesis System for reverse transcriptase-PCR (RT-PCR; Life Technologies, CA, USA).

3.5. PCR amplification of partial CDV nucleoprotein (CDV-N) and hemagglutinin genes (CDV-H)

cDNA was used as a template for conventional nested PCR using two sets of primers that amplified the nucleocapsid gene (*CDV-N*) [15]. For further confirmation, the whole hemagglutinin gene (*CDV-H*) was amplified using two pairs of primers [19]. We used the CDV vaccine (Boehringer Ingelheim) as the positive control and non-template master mix PCR as the negative control. PCR amplification was carried out on a thermal cycler machine (Veriti, Applied Biosystem). The amplified PCR product was analysed using 1.8% agarose gel electrophoresis. Electrophoresis was performed with SYBR Safe (Thermo Fisher) in tris acetic EDTA (TAE buffer). Using the Quantity One program, it was visualised on a GelDoc machine (BioRad).

3.6. Nucleotide sequencing and bioinformatic analysis

The PCR product was nucleotide sequenced in the 1st Base DNA sequencing service (Selangor, Malaysia) using two amplification direction primers. The nucleotide sequencing result was then analysed using

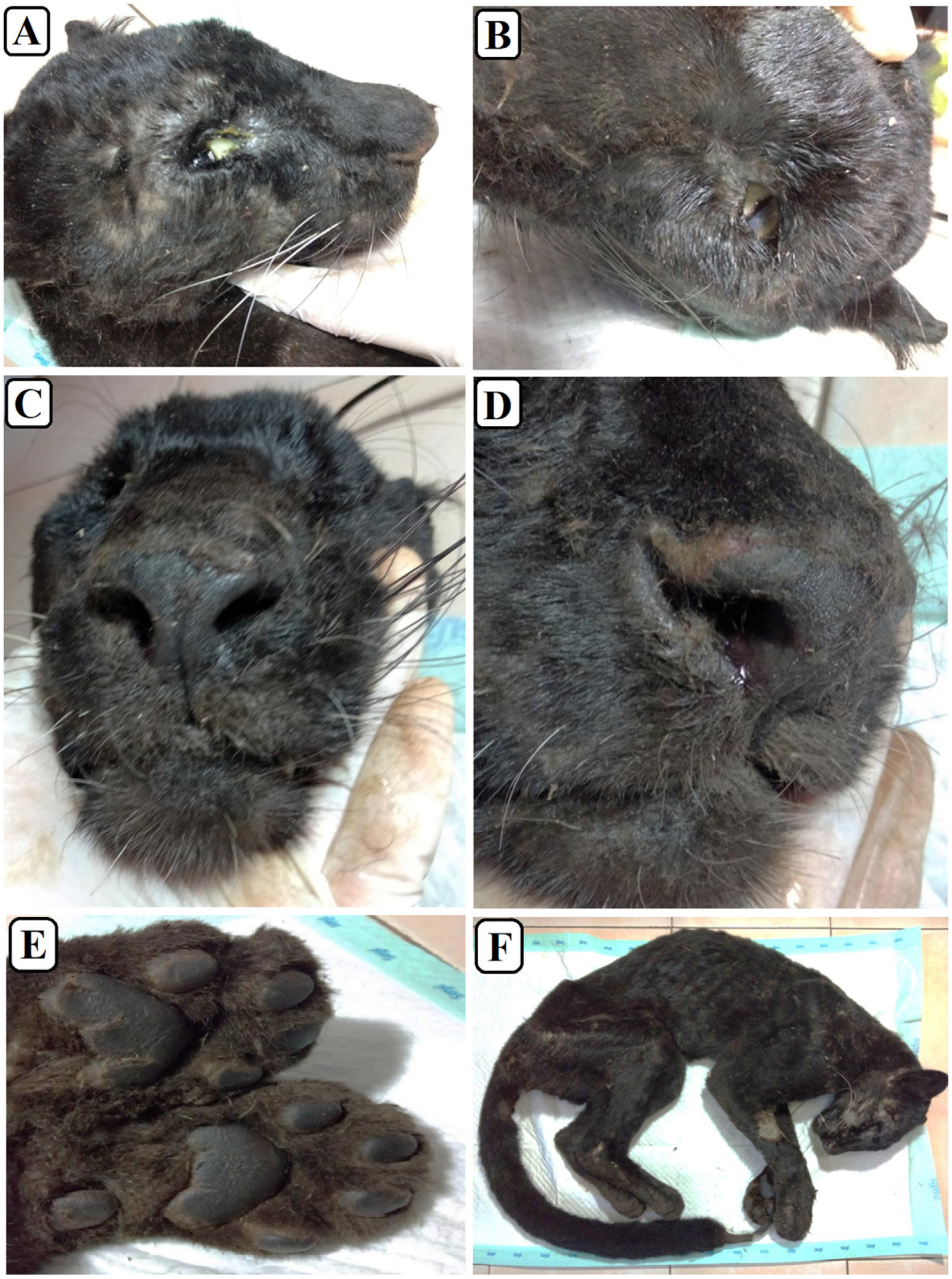

Figure 1. The Javan leopard 'Rita' post-mortem condition: (A) slight discharge from the right eye, (B) the left eye seems turbid, (C) dry nasal discharge around the nostrils, (D) slight bloody discharge in the right nostril, (E) hyperkeratosis on footpads–one of Canine Distemper's common symptoms-was found on all Rita's footpads, and (F) Rita's body condition after death.

the BioEdit program and aligned using NCBI Basic Local Alignment Search Tool (BLAST) program. The sequence was aligned with previously published CDV sequences deposited in GenBank using the ClustalW program. The accession numbers of all sequences are indicated in the taxon names. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model [20] in the 1000 repetition bootstrap test. The construction and analysis of phylogenetic trees were carried out in MEGA7.0 [21].

4. Results

4.1. Haematology and blood chemistry analysis

The haematological examination showed neutrophilia with an average maximum WBC count (WBC 20.8 × 103/μI; Neutrophils 19.5 × 103/μI). Blood chemistry showed hypoproteinemia with hypoglobulinemia (total protein 4.8 g/dl; Globulin 2.0 g/dl). Meanwhile, the urine analysis examination showed urine specific gravity of 1.025; pH 6; proteinuria 2+ (reference for haematology and blood chemistry using https://zims.species360.org).

4.2. PCR amplification and sequence characterisation of partial CDV nucleoprotein (CDV-N) and hemagglutinin (CDV-H) genes from Javan leopard

First, the faecal sample was analysed using RT-nested PCR amplification to detect the CDV RNA virus. We used two pairs of specific primers for the CDV nucleoprotein gene (CDV-*N*). Partial nucleotide sequences of *CDV-N* were determined using the direct sequencing method, resulting in 331 bp contig nucleotide sequences. The BLAST alignment (NCBI)

showed that our sequence was 99.97% similar to HQ850149 CDV isolated from domestic dogs in China.

We then amplified to 2029 bp of whole CDV-H using two pairs of primers from Javan leopard faecal and brain samples to characterise this CDV virus further. We used walking primer methods on the nucleotide sequences and obtained 807 bp nucleotides encoding 269 deduced amino acids (deposited in GenBank with accession number OM201303). The partial CDV-H sequences of this Javan leopard sample showed nucleotides and amino acid similarities at 97%–98% with other CDVs (Asia 1 genotypes group). This Asia 1 genotypes group include CDV dog/China (Genbank accession number FJ848533.1, HQ850147.1, JN896331, KJ489383.1), CDV fox/China (JX844219.1, FJ810215.1, JX681125.1), CDV mink/China (EU379560.1), CDV raccoon dog/China (KJ848781.1), and CDV giant panda/China (KP793921.1).

4.3. Phylogenetic analysis of partial CDV hemagglutinin (CDV-H) genes from Javan leopard

The phylogenetic tree was constructed based on 807 bp nucleotides of partial *CDV-H* to predict the evolutionary relationship of this Indonesian Javan leopard CDV sequence compared to the GenBank database's others (Figure 2). The result showed that the sequence was clustered with Asiatic CDV (Asia 1 genotype) such as dogs from China (FJ848533, HQ850147, JN896331, KJ489383), mink (EU379560), raccoon dog (KJ848781), fox (JX844129, FJ810215, JX681125), the giant panda (KP793921) and *Macaca fascicularis* (AB687720). The closest relationship of our sequence was to CDV fox (JX681125) with 98.7% identity. The phylogenetic tree results also indicated that our CDV sequence was separate in different clusters from other CDVs such as Asia 2, Asia 3, USA-Europe, Africa, and CDV vaccine sequences at 5.6%–11.6% nucleotide differences.

5. Discussion

Javan leopards are classified as Endangered by the International Union for the Conservation of Nature, with fewer than 500 individuals thought to exist in the wild [22]. Their remaining habitat is severely fragmented in 22 (84.3%, 9,748.25 km^2) of 29 predicted suitable landscapes (11,598.63 km^2), which represents only 8.9% of the total area of Java Island [23]. Many populations are small, isolated, and vulnerable to stochastic processes such as outbreaks of infectious diseases. Our findings are believed to be the first recorded case among the wild Javan leopard population and the second infection among Indonesian big cats after the Sumatran tiger (*Panthera tigris sumatrae*) [10]. Furthermore, this poses an additional threat to the already endangered Javan leopard population and other wildlife populations. Javan leopards are the last apex predator and flagship species remaining in Java after Javan tigers were declared extinct in the 1980s [24]. Leopards have been extirpated from 84% of

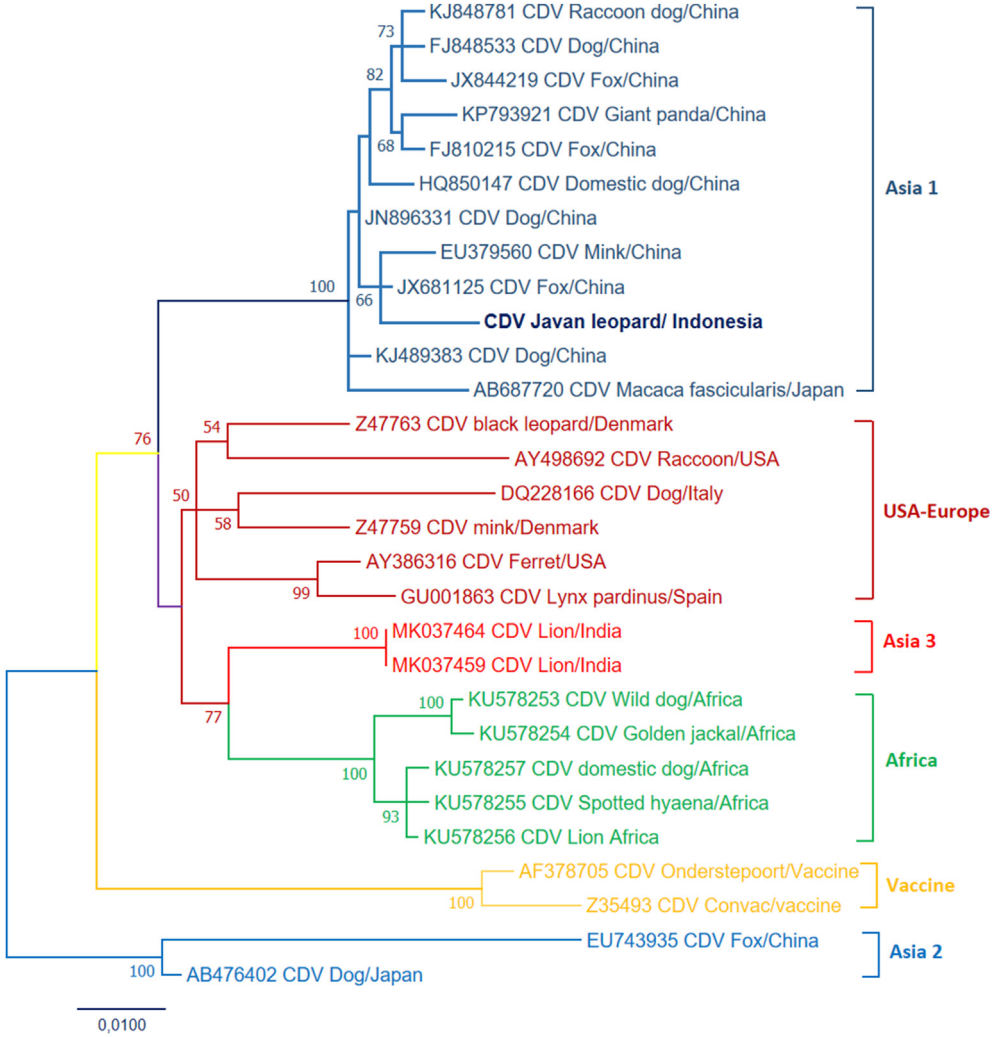

Figure 2. Phylogenetic tree analysis of the CDV partial hemagglutinin (*CDV-H*) gene of Javan leopard compared to other CDVs sequences in Genbank. The tree was constructed using MEGA 7.0 with the maximum likelihood method based on the Tamura-Nei model with 1000 replicate bootstrap. The analysis involved 29 sequences of a total of 807 contig nucleotides.

their historical range in Java [25] and now only persist in heavily fragmented forest patches [22].

This report describes an unusual death of a rescued wild Javan leopard (*Panthera pardus* ssp. *melas*), Rita, and our findings strengthen the CDV infection supposition in this case, similar to the CDV's Asia 1 strain. Our haematology and blood chemistry analysis show hypoproteinemia. Hypoproteinemia with normoalbuminemia indicates hypoglobulinemia, which is a common condition of a disorder or depression of the immune system. These findings strengthen our results of a CDV infection in Rita.

As a multi-host morbillivirus, CDV could infect all terrestrial carnivores [26], including the genus *Panthera* [27]. Out of curiosity, we also conducted molecular analysis for the feline panleukopenia virus using specific primers for the capsid gene. This analysis was undertaken since the infections of these two viruses can result in the same symptoms [28]. Since the feline panleukopenia virus molecular analysis of the samples was negative (data not shown), we are certain that Rita was infected by CDV.

Recently, CDV or antibodies to this virus has been reported in other species of wild cats, such as the endangered Amur tiger (*Panthera tigris altaica*) and the far east wildcat (*Prionailurus bengalensis euptilurus*) [29, 30]. Serological, demographic, and phylogenetic studies of dogs and wildlife populations in the Russian Far East show that several wildlife species are more important than dogs, both in maintaining CDV and as a source of infection for tigers [30].

Similar disease transmission may have occurred in within Java Forest habitats and wild species before our finding. Thus, investigating the appearance of the disease or the virus antibody in wildlife could help us increase our understanding of the virus transmission in the area. We encourage governments, institutions, and practitioners involved in conserving the Javan leopard and other wildlife species to increase public awareness. Personnel working with leopards should be aware of the possibility of future cases and, if possible, submit samples for antibody tests whenever leopards are handled.

Furthermore, we recommend implementing strict initial procedures for newly rescued wild animals placed in conservation institutions (including animal rescue centres). In principle, the initial steps that must be taken to emphasise the stable condition of the wildlife species in question are based on standardised animal welfare principles. By observing general animal data (e.g., species name, age, general condition), the stabilisation procedure aims to prevent animals from experiencing stress and deterioration to prevent a decline in health status and potential death. After stabilisation, general health checks by a team of wildlife veterinarians should include overall condition checks and screening for species-specific pathogenic agents and/or zoonotic agents so that accurate treatment plans can be implemented or the cause of death can be determined.

Declarations

Author contribution statement

All authors listed have significantly contributed to the investigation, development and writing of this article.

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Data availability statement

The data that has been used is confidential.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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