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NOTE

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New canine parvovirus 2a infection in an imported Asian small-clawed otter (*Aonyx cinereus*) in Japan

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ABSTRACT. Post-import from the Republic of Indonesia to Japan in 2017, two juvenile, captive bred Asian small-clawed otters (*Aonyx cinereus*) exhibited gastrointestinal symptoms, including vomiting, diarrhea, and hematemesis, and died. One of them was examined postmortem. Microscopically, the small intestinal mucosa was necrotic with crypts lined by regenerating large epithelial cells. A gastric cardiac mucosal ulcerative lesion containing fungal yeasts and pseudohyphae morphologically indicated *Candida* spp. The lymph nodes exhibited marked lymphoid depletion. Canine parvovirus 2 (CPV-2) was isolated from an oral swab, and virus protein 2 (VP2) gene sequencing revealed new CPV-2a. To our knowledge, this is the first new CPV-2a infection report in Asian small-clawed otters. This infection should be considered in gastrointestinal symptom-related cases in this species.

KEY WORDS: *Aonyx cinereus*, Asian small-clawed otter, canine parvovirus 2, viral host range

The Asian small-clawed otter (*Aonyx cinereus*) belongs to the order *Carnivora* and family *Mustelidae*. These otters are semi-aquatic mammals native to South and Southeast Asia but are often kept in zoologic collections and as pets [9]. This species is considered "vulnerable" on the International Union for Conservation of Nature (IUCN) Red List. In addition, the species was listed in Appendix I by the Convention on International Trade in Endangered Species (CITES) in 2019, and the trade of these animals for commercial purposes was banned [3].

Canine parvovirus 2 (CPV-2; family *Parvoviridae*, genus *Protoparvovirus*, species *Carnivore protoparvovirus* 1) causes gastrointestinal disease in domestic and non-domestic carnivore species. It is a highly contagious pathogen that causes a fatal disease in infected dogs [13]. CPV-2 suddenly emerged in the late 1970s from feline panleukopenia virus or related carnivore parvoviruses [4, 25]. Shortly after its emergence, CPV-2 continued to evolve, and various antigenic variants cocirculated among domestic dogs [19, 20]. In addition, CPV infection has already been demonstrated in the *Canidae*, *Felidae*, *Procyonidae*, and *Mustelidae* families [1, 24].

The substitution of a few amino acids in virus protein 2 (VP2), the main viral protein of the icosahedral capsid, alters the relevant biological characteristics of the virus [5]. In the following years, the original CPV-2 was replaced worldwide by three different genetic variants known as CPV-2a, CPV-2b, and CPV-2c, which only differ from the original virus at a few amino acid residues [2]. Subsequently, the amino acid residue at position 297 of VP2 changed from Ser to Ala, which was detected in prototype CPV-2a/2b viruses in Europe, and they were called new CPV-2a and -2b [12, 18]. The sequence analysis of the VP2 gene of CPV-2 and subsequent characterization is important for the determination of antigenicity and viral host ranges [14]. Here, we aimed to describe a fatal case of new CPV-2a infection in an Asian small-clawed otter in Japan.

Two juvenile captive bred Asian small-clawed otters (1 male and 1 female) were legally imported from the Republic of Indonesia to Japan on November 11, 2017. Post-import, they developed gastrointestinal symptoms and eventually died. On the day of import, the 780 g, 108-day-old female otter exhibited depression, diarrhea, and vomiting. At 3 days post-import (dpi), the otter was taken to a veterinary clinic and treated subcutaneously with the antibiotic cefovecin sodium (8 mg/kg, SC, Convenia;

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Zoetis, Tokyo, Japan), followed by toltrazuril (15 mg/kg, PO, Baycox; Bayer Pharmaceuticals, Osaka, Japan) and lactated Ringer solution (30 ml/kg, SC). The otter died at 5 dpi. At 4 dpi, the 390 g, 55-day-old male otter exhibited conjunctivitis and diarrhea and was taken to a veterinary clinic and subcutaneously treated with the antibiotic enrofloxacin (5 mg/kg, SC, Baytril; Bayer Pharmaceuticals) and 15 ml/head of lactated Ringer solution. At 11 dpi, the otter exhibited episodes of vomiting and hematemesis, and the antiemetic maropitant (1 mg/kg, SC, Cerenia; Zoetis), was administered; however, the animal subsequently died. Endoparasites were not detected in any of the fecal samples. The male otter was submitted for a postmortem examination.

Tissue samples (brain, lung, trachea, bronchi, heart, esophagus, liver, stomach, intestine, spleen, and kidneys) were fixed in 10% neutral-buffered formalin, routinely processed, and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) stain for a histological examination. Oral, rectal, and nasopharyngeal swabs were collected from the otter for molecular and virologic testing.

To detect the VP2 gene of CPV-2, deoxyribose nucleic acid (DNA) was extracted from the swabs (DNeasy blood & tissue kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. The VP2 gene was amplified by PCR (Ex Taq; Takara, Kusatsu, Japan). PCR was performed using the primer pairs, 555F (5′-CAGGAAGATATCCAGAAGGA-3′) and 555R (5′-GGTGCTAGTTGATATGTAATAAACA-3′) [2]. PCR was performed at 94°C for 2 min, followed by 40 cycles at 98°C for 10 sec, 50°C for 30 sec, 72°C for 1 min and final extension at 72°C for 10 min. PCR products were electrophoresed on a 2.0% agarose gel. To determine the complete nucleotide sequences of the VP2 gene of CPV-2, the VP2 gene was amplified by PCR (Ex Taq; Takara). PCR was performed using the primer pairs, VP2FLf (5′-GTGCAGGACAAGTAAAA-3′) [6] and 555R (5′-GGTGCTAGTTGATATGTAATAAACA-3′) [2]. PCR was performed at 94°C for 2 min, followed by 40 cycles at 98°C for 10 sec, 50°C for 30 sec, 72°C for 2 min and final extension at 72°C for 10 min. PCR products were purified (MinElute PCR Purification Kit; Qiagen, Hilden, Germany). The nucleotide sequences were determined using the BigDye terminator v.3.1 cycle sequencing kit (Thermo Fischer Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

A72/cSLAM cells expressing the canine signaling lymphocyte activation molecule (SLAM) [17] were grown in Dulbecco modified Eagle medium (DMEM; Thermo Fisher Scientific) containing 10% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Thermo Fisher Scientific). Vero cells (Vero 9013, JCRB9013) purchased from the Health Science Research Resource Bank (HSRRB, Japan) were cultured in Eagle's minimum essential medium (EMEM; Thermo Fisher Scientific) containing 5% FCS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

The sample mixture was centrifuged at 2,000 × *g* for 15 min at 4°C. The supernatants were filtered through 0.45-µm centrifuge tube filters (Costar Spin-X; Corning, Corning, NY, USA). The filtrates were inoculated on A72/cSLAM and Vero cells. The cells were incubated with DMEM or EMEM containing 2% FCS and a 1% antibiotic-antimycotic solution (Thermo Fisher Scientific) at 37°C. The cells were maintained and passaged until a cytopathic effect (CPE) was observed. After five blind passages, the samples without a CPE were deemed as negative for virus isolation.

Upon autopsy, there was a large amount of tarry material in the oral cavity. The gingival mucosa was diffusely pale, which is suggestive of severe anemia (Fig. 1a). The thymus was very small. The intestine was flaccid, and the mesenteric adipose tissue was indiscernible. The spleen was atrophied. The mesenteric lymph node was inconspicuous compared to those of age-matched animals (Fig. 1b). The lungs failed to collapse and were edematous. There was a 10 × 4 mm ulcer in the cardiac mucosa of the stomach (Fig. 1c, arrow and inset). The femoral bone marrow was red.

Microscopically, the small intestinal mucosa was necrotic with crypts lined by regenerating large epithelial cells. The remaining crypt epithelial cells have small intranuclear inclusion bodies (Fig. 2a). There was an ulcerative lesion in the gastric cardiac mucosa containing fungal yeasts and pseudohyphae (2–5 µm in diameter), which was morphologically suggestive of *Candida* spp. (Fig. 2b). There was marked lymphoid depletion in all the lymph nodes (Fig. 2c). The spleen was characterized by a marked lymphoid depletion in the white pulp. The bone marrow contained decreased numbers of hematopoietic cells of all three lineages, thereby highlighting the background plasma cells (Fig. 2d).

The VP2 capsid protein-encoding gene of CPV-2 was detected in all the swabs, and CPV-2 was isolated from the oral swab using A72/cSLAM cells. The isolated virus was named Pr17137O-CPV2, and the amino acid sequence of the VP2 gene was determined and deposited into the DNA Data Bank of Japan (DDBJ) (accession no. LC570804), thereby indicating that the isolated virus was a new CPV-2a.

CPV has an affinity for the rapidly dividing cells of the intestine and causes acute enteritis with intestinal crypt necrosis and villus atrophy. The virus also has tropism for the bone marrow and lymphoid tissues; thus, leukopenia and lymphoid depletion accompany intestinal destruction [16]. Based on the clinical course, histologic findings, and molecular test results, a clinical diagnosis of CPV infection was made in the male otter. Bleeding as a result of the gastric ulceration was associated with vomiting due to CPV infection, which resulted in anemia that may have been the primary cause of death. Parvovirus-induced disease could cause a predisposition to secondary infections, potentially increasing mortality rates [26]. *Candida* spp. normally inhabit the mucosa of the gastrointestinal, upper respiratory, and genital tracts of mammals. Candidiasis associated with parvoviral infection has been reported in a puppy [22]. In our case, we considered that fungal yeast infection was the most likely opportunistic invasion that occurred secondary to the gastric mucosal injury caused by CPV-2 infection due to immunosuppression.

The different antigenic variants of CPV-2 are prevalent in varying proportions in Asian countries. The predominant CPV epidemic strains, the new CPV-2a and CPV-2c, have exhibited significant growth trends since 2010 in China [21]. The first case involving CPV-2c was detected in 2015, and it has since become the predominant strain in Taiwan [11]. Currently, the predominant antigenic variants of CPV-2 detected in dogs with either acute hemorrhagic diarrhea or diarrhea in Thailand are new CPV-2a and CPV-2c [10]. CPV-2c is the most prevalent variant in South Korea [15]. CPV-2b was more predominant than CPV-2a in

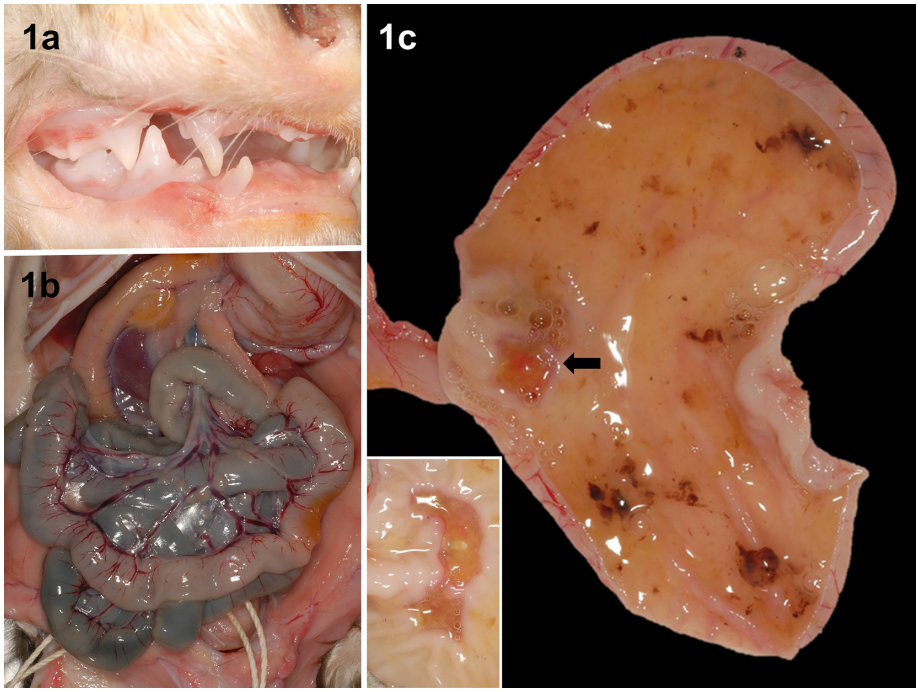

Fig. 1. Gross lesions of an Asian small-clawed otter (*Aonyx cinereus*) infected by new canine parvovirus 2a. (a) The gingival mucosa was diffusely pale, suggestive of severe anemia. (b) The intestine was flaccid, and the mesentery was devoid of fat. The mesenteric lymph node was inconspicuous for the animal's age. (c) The cardiac mucosa of the stomach had a 10 × 4 mm ulcer (arrow). The inset shows a magnified view of the ulcer.

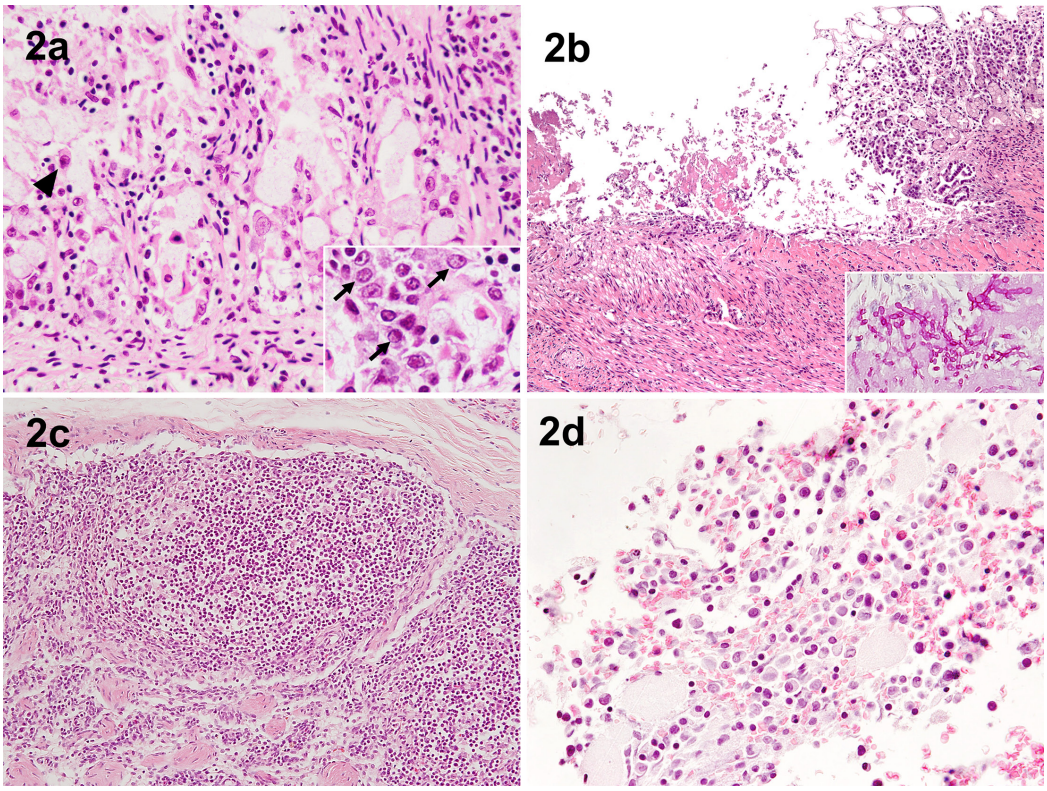

Fig. 2. Hematoxylin and eosin staining of microscopic lesions of an Asian small-clawed otter (*Aonyx cinereus*) infected by new canine parvovirus 2a. (a) The degenerative and necrotic enterocytes characteristic of parvoviral necrotic enteritis. Small intestinal mucosal crypts are lined by regenerating large epithelial cells. The arrowhead points to a large cell with bizarre nucleus. The remaining crypt epithelial cells have small intranuclear inclusion bodies (arrows). Hematoxylin and eosin staining. (b) The ulcerated gastric cardiac mucosa is covered by fungal organisms. Hematoxylin and eosin staining. The inset shows periodic acid-Schiff positive fungal yeasts and pseudohyphae morphologically suggestive of *Candida* spp. (c) The lymph node shows marked lymphoid depletion. Hematoxylin and eosin staining. (d) The bone marrow contains decreased numbers of hematopoietic cells of all three lineages, thereby highlighting the background plasma cells.

domestic dogs in Japan, and there have been no significant changes in the CPV-2 epidemic in recent years [7, 18, 23]. To the best of the author's knowledge, there have been no reports on the VP2 gene sequences of CPV-2 in Indonesia in the English-language literature to date.

The incubation period for CPV-2 is commonly 3–7 days [16]. The two juvenile Asian small-clawed otters, born at a breeding farm in Indonesia, had no contact with any other carnivores since their import to Japan. The otters exhibited clinical signs on the day of the import and 4 dpi, respectively. Based on the antigenic variants and incubation period of CPV-2, it is most likely that the male otter could have been exposed to CPV-2 in Indonesia, which then progressed to the developed clinical course after the import into Japan.

CPV-2a or CPV-2b infections have been described in Asian small-clawed otters in the US [8, 26]. To the best of the author's knowledge, this is the first report of new CPV-2a infection in Asian small-clawed otters, and new CPV-2a infection should be considered in cases involving gastrointestinal symptoms in this otter species. Our findings imply that Asian small-clawed otters are very susceptible to CPV-2.

The further accumulation of case studies is needed to understand the relationship between CPV antigenic variants and the host ranges of CPV. In addition, this case indicates that international live animal trade plays a role in the spread of viral pathogens.

CONFLICT OF INTEREST. The authors have no conflicts of interest directly relevant to the content of this article.

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