1 NOTE

2 Virology

3 Identification of domestic cat hepadnavirus from a cat blood sample in Japan

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26 Running head: IMPACT OF DCH ON HEPATIC DISEASES IN DOMESTIC CATS

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28 **ABSTRACT**

29 The hepatitis B virus (*Hepadnaviridae*) induces chronic hepatitis and hepatic cancer in humans. A
 30 novel domestic cat hepadnavirus (DCH) was recently identified in several countries, however, the
 31 DCH infection status of cats in Japan is unknown. Therefore, we investigated the DCH infection rate
 32 of 139 cat samples collected in Japan. We identified one positive blood sample (0.78%) from a 17-
 33 year-old female cat with chronically elevated alanine aminotransferase. Phylogenetic analysis
 34 demonstrated that the DCH strain identified in this study is genetically distinct from strains in other
 35 countries. Further investigations are required to elucidate the evolution of DCH and the impact of
 36 DCH infection on hepatic diseases in domestic cats.

37

38 **KEY WORDS:** domestic cat hepadnavirus (DCH), hepatic virus, pet cats

39 Main text

40 Hepatitis B virus (HBV) (*Hepadnaviridae*) is one of the causative agents of chronic hepatitis and
41 hepatocellular carcinoma. Despite effective vaccines against HBV, over 350 million people worldwide
42 are chronically infected with HBV [1]. One step in the life cycle of HBV involves the conversion of
43 viral pregenomic RNA to a relaxed circular DNA; therefore, several reverse transcriptase inhibitors in
44 combination with interferons are currently used for controlling HBV infection [2].

45 It has been unclear whether other mammals can be infected by similar hepadnaviruses that
46 induce chronic hepatitis and hepatocellular carcinoma. In 2018, an Australian group identified a novel
47 feline hepadnavirus, the domestic cat hepadnavirus (DCH), and since then, it has been detected in cats
48 in several countries including Australia (6.5% of tested samples) [3] and Italy (10.8% of tested
49 samples) [4]. Although it remains unclear whether DCH can induce hepatic diseases in cats, the
50 detection of DCH has been associated with chronic hepatitis and hepatocellular carcinoma [5]. In
51 addition, cats infected with the feline immunodeficiency virus (FIV) also tend to be positive for DCH
52 [3], suggesting that immunodeficiency induced by FIV may lead to infection with DCH.

53 The fact that DCH has been discovered in several countries suggests that it is prevalent
54 worldwide. Phylogenetic analyses in previous studies suggest that DCH is divergent among strains
55 from different countries, suggesting that DCH has been evolving in each country. So far, DCH has
56 been identified Australia [3], Thailand [6], Italy [5], the United Kingdom [7], and Malaysia [8]. Its
57 presence and prevalence in Japan, however, remains undetermined. In this study, therefore, we
58 investigated the presence and prevalence of DCH in Japan.

59 We screened 139 blood samples collected from several clinics in Japan. The blood samples
60 were collected from cats that were mainly housed indoors. We used samples that were rest of blood
61 testing for diagnosis. Prior to testing for DCH, we obtained the owner's informed consent. This study
62 was approved by the Animal Experiment Committee of the University of Miyazaki (authorization
63 number: 2021-019). All experiments were performed in accordance with relevant guidelines and
64 regulations.

65 All collected blood samples were mixed with the anticoagulants, heparin or ethylenedinitrilo-

66 tetraacetic acid disodium (EDTA/2Na). Previous studies have demonstrated that heparin inhibits PCR [9], thus
 67 we used a KOD One PCR Master Mix (TOYOBO, Osaka, Japan), which is relatively tolerant to heparin
 68 contamination. To check the quality of the PCR, we used primer pairs to amplify the cat glyceraldehyde-3-
 69 phosphate dehydrogenase (*Gapdh*) housekeeping gene. The primers used to amplify the DCH genome are
 70 DCH-F: (5'-ATTCAAGCGCTCTATGAAGAGG-3') and DCH-R: (5'-AAAAGTGAGGCAAGAGAGATGG-
 71 3'), while those used to amplify cat *Gapdh* are GAPDH-F: (5'-CCTTCATTGACCTCAACTACAT-3') and
 72 GAPDH-R: (5'-CCCCAGTAGACTCCACAACATAC-3'). The PCR conditions were 40 cycles of 98°C for
 73 10 sec, 60°C for 5 sec, and 68°C for 10 sec, followed by 68°C for 7 min. We used a synthesized DNA
 74 fragment encoding the partial DCH genome (Eurofins) as a positive control for DCH. The amplicons were
 75 visualized on a 1.5% agarose gel.

76 We detected *Gapdh* amplicons from the 129 samples tested. A single sample (#116) (0.78% of
 77 tested samples) was positive for DCH (**Figure 1**). We then sequenced the entire viral genome using
 78 six pairs of primers whose sequences are listed in **Table 1**. We designed the primers on the Primer3
 79 website (<https://bioinfo.ut.ee/primer3-0.4.0/>). All six fragments were efficiently amplified. Each
 80 amplicon was run on an agarose gel and then extracted from the gel with QIAquick Gel Extraction Kit
 81 (Qiagen, Tokyo, Japan). The sequences of the amplicons were determined using a BigDye Terminator
 82 v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an Applied Biosystems 3130xl DNA
 83 Analyzer (Thermo Fisher Scientific). The sequence assembly was performed with 4Peaks
 84 (Nucleobytes) and Microsoft Word 2019 (Microsoft). The assembled sequence was deposited in
 85 GenBank (Accession# LC668427).

86 The sequence of our strain was aligned with those of 15 other DCH strains and the sequences
 87 were analyzed phylogenetically using MEGA X (MEGA Software). Our phylogenetic analysis
 88 revealed that the amino acid sequences of the polymerase, surface, and core proteins of Domestic cat
 89 hepadnavirus Japan/KT116/2021 were genetically close to those of previously reported strains
 90 (**Figure 2**). In contrast, the amino acid sequence of the X protein of Domestic cat hepadnavirus
 91 Japan/KT116/2021 was distinct from those of other strains. The X protein of HBV (HBx) plays roles

92 in silencing host antiviral defenses and promoting viral transcription [10, 11]. Therefore, it will be
93 important to elucidate the impact of polymorphism in the DCH X protein.

94 The source of the DCH-positive sample (#116) was a 17-year-old female cat (Cat #116) born
95 in Japan and with no record of traveling overseas. Therefore, Cat #116 was likely infected with DCH
96 in Japan. At the time of the PCR test, Cat #116 had no health problems, based on interviews and
97 physical examinations. However, prior to its death due to acute neuropathy, we observed a persistent
98 elevation of alanine aminotransferase (ALT) (**Figure 3**). Moreover, Cat #116 underwent a
99 splenectomy one year before the PCR test, and was then diagnosed with a mast cell tumor. After this
100 diagnosis, the cat was treated with CCNU, an anticancer drug. While we cannot exclude the
101 possibility that the treatments against the mast cell tumor induced elevated levels of ALT, it is also
102 possible that DCH infection had affected the health status of Cat #116. Currently, it is not known
103 whether the mast cell tumor itself or the treatments against mast cell tumor had induced
104 immunosuppression in Cat #116, leading to infection with DCH. As Cat #116 was tested positive for
105 DCH after its death, we were unable to perform an autopsy. Note that Cat #116 was negative for FIV
106 and feline leukemia virus (FeLV).

107 In conclusion, to the best of our knowledge, this is the first time that DCH has been detected
108 in a cat in Japan. The prevalence of DCH in this study (0.78%) is lower than those of previous studies
109 in other countries [3, 4]. Further investigation is required to determine the reason for this difference.
110 The results of phylogenetic analysis of the X protein reveal that Domestic cat hepadnavirus
111 Japan/KT116/2021 is genetically distinct from previously described strains from other countries,
112 suggesting that Domestic cat hepadnavirus Japan/KT116/2021 is a strain native to Japan. Because
113 homologous recombination has been reported with HBV [12, 13] or DCH [6], it is important to
114 monitor the infection status and evolutionary history of DCH in every country with a large domestic
115 cat population. Also, the impact of DCH infection on chronic hepatitis in cats should be elucidated.

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119 **CONFLICTS OF INTEREST**

120 The authors declare no conflict of interest.

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122 **ACKNOWLEDGMENTS**

123 This work was supported by grants from Japan Society for the Promotion of Science (JSPS)

124 KAKENHI Grant-in-Aid for Scientific Research (C) 19K06382 (to AS); KAKENHI Grant-in-Aid for

125 Scientific Research (B) 21H02361 (to TO and AS); and from a Grant for Joint Research Projects of

126 the Research Institute for Microbial Diseases, Osaka University (to AS). We thank Tomoko Nishiuchi

127 for her support.

128

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Figure legends

Fig. 1. PCR screening of blood samples from cats to detect the domestic cat hepadnavirus (DCH) genome

Heparinized blood was used to amplify DCH and the cat *Gapdh* gene. Marker, N.C., and P.C. denote DNA size marker, negative control, and positive control, respectively.

Fig. 2. Phylogenetic analysis of DCH/Japan/KT116/2021

The phylogenetic position of DCH Malaysia within the family *Hepadnaviridae*. The maximum likelihood tree is based on the each hepadnaviral protein sequences retrieved from the GenBank database. The Domestic cat hepadnavirus Japan/KT116/2021 (Accession# LC668427) is indicated by a red dot. The tree is drawn to scale, thus the branch lengths correspond to the number of substitutions per site.

Fig. 3. Changes in alanine aminotransferase (ALT) levels in Cat #116

A plot of ALT levels in each blood test. The X-axis denotes “Days after PCR test.”

Figure 1

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Figure 2

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Polymerase protein

Surface protein

Core protein

X protein

Figure 3

1 **TABLE 1. Primers used for determining viral whole genome**

Fragment	Forward primer	Reverse primer
#1	5'-ACTCTCAAACAGGGAACATTCGT-3'	5'-CATCCGACCGGAATAATAATTAAC-3'
#2	5'-AATTCTCCAAAGGCTAACAGGTTTA-3'	5'-ATTCCACCAATAGCAGATCACGTAG-3'
#3	5'-TACGTCCCTTCCACTCTGAATC-3'	5'-CAAGACAGTATGTTGTCCAAAAGTG-3'
#4	5'-GAAGAGGAACTTACAGGTAGGGAAC-3'	5'-GTCTAGATTGTGACGAGGGAAAAAC-3'
#5	5'-CTCGATACCCTGATTATTCTCTTCA-3'	5'-CCCTATTGTTTGTATTTTGTCCAC-3'
#6	5'-CAGTTGGAGACAGAAGTACGGTTAT-3'	5'-CATCCATATAAGCAAACACCATACA-3'

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