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Detection and genetic characterization of domestic cat hepadnavirus in cats with cavitary effusions

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ABSTRACT

After the identification of the novel domestic cat hepadnavirus (DCH) in 2018, its potential pathogenetic role in feline hepatic diseases has been suggested. Following the detection of DCH in a cat's serum and peritoneal effusion, the aim of this study was to retrospectively investigate the presence of DCH in cats with and without cavitary effusions along with DCH presence in effusions. Stored serum and effusion samples from cats with and without effusions admitted to the Veterinary Teaching Hospital of Lodi (Italy) in 2020–2022 were included based on results of hematobiochemical parameters. Effusions were classified based on cytological and physicochemical findings. The likelihood of liver damage was estimated based on clinical and laboratory findings. Samples were tested for DCH presence by quantitative PCR (qPCR). Positive samples were subjected to whole genome sequencing and phylogenetic analysis. DCH was detected in both serum and peritoneal effusion samples of 2/72 (2.8%) enrolled cats, included in the group with effusions (2/33; 6.1%), with one cat showing inflammatory and the other non-inflammatory effusion. Both DCH-positive cats belonged to the group with a likelihood of liver damage (2/22, 9.1%). Phylogeny showed that the DCH sequences from this study clustered with the prototypic Australian strain but were not included in the clade with other Italian DCH sequences. Results suggest the circulation of different DCH variants in Italy and show the presence of DCH in effusion samples from DCH-positive cats, mirroring the presence of HBV in body fluids from HBV-infected humans. Further studies are still recommended to define the pathogenic role of DCH in cats.

1. Introduction

Viruses of the genus *Orthohepadnavirus*, family *Hepadnaviridae*, are partially double-stranded DNA viruses reported in different mammal species, including bats, primates and rodents (Seeger et al., 2013). The prototype species of the genus, Hepatitis B virus (HBV), is a major global health problem causing potentially life-threatening liver infections in humans. In 2019, HBV infections resulted in an estimated 820,000 deaths, mostly from cirrhosis and hepatocellular carcinoma (HCC) (Seeger et al., 2013; WHO, 2022). In humans, HBV and human immunodeficiency virus (HIV) co-infections have been commonly reported (Petty et al., 2014).

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In 2018, a novel divergent member of the genus *Orthohepadnavirus,* named domestic cat hepadnavirus (DCH), was identified during transcriptomics studies in an Australian cat affected by a multicentric large B-cell lymphoma and feline immunodeficiency virus (FIV) infection. Whole genome sequencing revealed a DCH genome of approximately 3.2 kb containing four overlapping open reading frames (ORFs), which encode for the surface (S), core (C), polymerase (P) and X proteins (Aghazadeh et al., 2018). Since its discovery in Australia, the presence of DCH in blood or serum samples of both healthy and unhealthy cats has been reported in other countries including Thailand, Malaysia, China, Japan, Italy, the United Kingdom and the USA, with prevalence ranging from 0.2% to 12.4% (Aghazadeh et al., 2018; Lanave et al., 2019; Piewbang et al., 2020; Anpuanandam et al., 2021; Jeanes et al., 2022; Scavone et al., 2022; Stone et al., 2022; Takahashi et al., 2022; Capozza et al., 2023). Moreover, a 25% seroprevalence has been reported in cats in Italy using an antibody detection enzyme-linked immunosorbent assay based on the recombinant core antigen of DCH (Fruci et al., 2022). DCH presence has been associated with feline retroviruses (FIV and/or feline leukemia virus, FeLV) infections, adult age and type of ownership, with higher detection of DCH among pet cats compared to shelter cats (Aghazadeh et al., 2018; Lanave et al., 2019; Piewbang et al., 2020; Anpuanandam et al., 2021; Piewbang et al., 2022).

The potential pathogenetic role of DCH in feline liver diseases has been recently investigated. In an international multicenter investigation, DCH infection has been associated with HCC and chronic hepatitis in cats, with histopathological features of inflammation, neoplasia and a viral distribution highly similar to that seen with HBV-associated disease in humans (Pesavento et al., 2019). DCH has recently been also associated with chronic lymphoplasmacytic hepatitis in cats (Piewbang et al., 2022). Indeed, cats with DCH viraemia are likely to have elevated serum alanine transaminase (ALT) activity, suggestive of liver injury (Lanave et al., 2019; Anpuanandam et al., 2021; Capozza et al., 2021a; Piewbang et al., 2022). However, in a recent study on cats with low, intermediate or high likelihood of liver disease based on the presence/absence or the magnitude of changes in liver enzymes, DCH was detected in the serum of individuals with normal or mildly elevated liver enzymes but not in the serum from cats with a high likelihood of liver disease (Scavone et al., 2022). Besides liver diseases, a study described DCH systemic infection in three cats, associated with inflammatory lesions in various organs including the liver, kidney, intestine, heart and lungs (Piewbang et al., 2020).

Studies to understand DCH infection in cats are warranted (Capozza et al., 2021a; Shofa et al., 2022). In November 2021, in the framework of a screening of pathogens carried out at the Veterinary Teaching Hospital (VTH) of Lodi (Italy), DCH was detected by quantitative real-time PCR (qPCR) (Lanave et al., 2019) in serum and peritoneal effusion samples of a cat with a history of hyperthermia, diffuse oedema, suspected liver disease and cavitary effusions of unknown etiology. Following this finding, this retrospective study aimed to investigate the presence of DCH infection in serum samples from cats with and without effusion and the presence of DCH in cavitary effusions to provide additional information about DCH infection and viral variants in cats.

2. Materials and methods

2.1. Animals and samples selection

Cats with or without cavity effusions from the Lombardy region, Northern Italy, admitted to the VTH of Lodi between January 2020 and March 2022 were randomly and retrospectively selected for this study. Criteria of inclusion were the availability of at least one residual leftover serum sample for molecular analysis, the availability of the results of ALT and alkaline phosphatase (ALP) activity measured at admission during the initial diagnostic work-up and the analysis of effusion (if present). Archive samples were stored at − 20 °C. According to the Institution guidelines, formal approval of the Ethical committee was not required because samples used in this study were obtained from leftover samples collected for diagnostic purposes or as part of health checks and with the informed consent of the owners (decision no. 2/2016 of the Ethical Committee of the University of Milan). Data regarding signalment, clinical history (with special emphasis on the presence or absence of cavitary effusion) and laboratory findings on serum samples and cavitary effusions recorded for each cat were retrieved from the laboratory database. The cats were categorized into two age groups: < 2 and ≥ 2 years, as previously reported (Anpuanandam et al., 2021). Regarding laboratory findings, results of biochemistry analyses performed on serum samples with an automated chemistry analyzer (BT 3500, Biotecnica Instruments, Rome, Italy) and reagents provided by the instrument's manufacturer were retrieved. Results of FeLV p27 antigen and FIV antibody presence detected using a commercially available kit (SNAP FIV/FeLV Combo, IDEXX Laboratories, USA) were recorded. When available, results of post-mortem examination of cats were also retrieved.

Moreover, the analysis of effusions, if present, included: 1) the physicochemical analysis of the fluid, which in turn included the visual evaluation of the gross appearance of the fluid, the measurement of the specific gravity and total solids using a portable refractometer (Sper Scientific, Scottsdale AZ, USA), the measurement of total proteins and of additional analytes such as creatinine, urea, potassium, cholesterol, triglycerides, when the clinical presentation raised the suspicion of uroperitoneum or of chylous effusion, using the chemistry analyzer mentioned above; 2) the total cell count measured using a laser-based analyzer (Sysmex XN-V, Sysmex Europe, Norderstedt, Germany); 3) cytology performed on smears and/or on cytocentrifuged fluids stained with a rapid stain (Hemacolor, Merck, Darmstadt, Germany); 4) results of bacteriology and/or PCR diagnostic analysis.

The enrolled cats were further divided into two groups based on the presence or absence of cavitary effusions. Cats with cavitary effusions were then categorized based on the localization of effusion and the type of effusion. To achieve this latter classification, samples were first classified based on physicochemical and cytological features, according to the main classification schemes (Stockham and Scott, 2008; Dempsey and Ewing, 2011) and then integrated with clinical history and results of bacteriology, PCR and, if present, necropsy report. For this study, the types of effusion were clustered into two main categories: inflammatory effusions (septic effusions, non-specific exudates and FIP effusions) and non-inflammatory effusions (neoplastic, hemorrhagic or chylous effusions, transudates, uroperitoneum).

Cats were also categorized according to biochemical analysis results for the likelihood of liver damage based on the blood activity of ALT and ALP as previously reported (Scavone et al., 2022) and/or total bilirubin concentration. The three categories of the likelihood of liver damage were the following:

- low: less than a two-fold increase in ALT activity without other enzyme abnormalities or more than two-fold increase in ALT activity paired with the simultaneous increase in CK activity and normal ALP, GGT activity and bilirubin concentration, suggestive of muscle damage or increase in ALP activity presumably due to isoenzymes of non-hepatic origin, possibly related to hyperthyroidism, without other enzyme abnormalities
- intermediate: more than two-fold and less than five-fold increase in ALT activity without changes in CK or less than two-fold simultaneous increase in ALT and ALP activity without other changes potentially consistent with hepatic disease and with high bilirubin (not associated with hematological changes consistent with hemolysis or with changes suggestive of cholestasis) or hyperbilirubinemia and other abnormalities consistent with liver failure (e.g. hypoalbuminemia or low urea and glucose level) even in absence of increased enzyme activity

– high: more than two-fold simultaneous increase in ALT and ALP activity without changes in CK or more than five-fold increase in ALT activity alone, independent of other enzyme abnormalities.

2.2. DNA extraction, PCR protocols and sequencing

DNA was extracted from serum and effusion samples using a commercial DNA extraction kit (NucleoSpin Blood kit, Macherey-Nagel, Germany), following the manufacturer's instruction. DNA was extracted from liver biopsies, if available, following routine diagnostic procedures, using a commercial DNA extraction kit (NucleoSpin Tissue kit, Macherey-Nagel, Germany), following the manufacturer's instruction. DNA pre-analytical quality control targeting vertebrate 12 S rRNA gene was performed on randomly selected samples (Kitano et al., 2007). All samples were subjected to a qPCR specific for DCH (Lanave et al., 2019) using a QuantStudio™ 3 instrument (Applied Biosystems, MA, USA). For DCH quantification, a 105 bp fragment of the Australian DCH reference strain AUS/2016/Sydney genome (GenBank accession no. MH307930) was cloned into a pGEM-T Easy plasmid vector (Promega, WI, USA) to produce a standard for the qPCR. The DCH copy number in each sample was calculated based on the standard curve generated from 10-fold dilutions of the plasmid prepared between 10^9 and 1 copies/μL. DCH DNA extracted from a naturally infected cat was used as positive control and a blank control (DNase-free water sample) was also included in all the qPCR reactions.

2.3. Whole genome sequencing of DCH positive samples

To characterize the genome of DCH stains detected in this study, a six primers-set PCR (Table 1) was designed based on the available DCH sequences deposited in GenBank. PCRs were performed using Phire Green Hot Start II PCR Master Mix (Thermo Scientific™, Vilnius, Lithuania) previously used for viral genome sequencing (Lin et al., 2016) in a final volume of 30 μL containing 1 μM of each primer and 5 μL (100–500 ng) of DNA template. Amplifications were carried out as follows: initial denaturation at 98 °C for 30 s, 40 cycles of 98 °C for 5 s, 54 °C for 5 s and 72 °C for 15 s; and a final elongation step at 72 °C for 1 min

The amplicons were purified using a commercial kit (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel, Germany) following the manufacturer's instruction and Sanger sequenced (Microsynth Seqlab, Germany) using the forward and reverse primers used for DNA amplification. The overlapping PCR products were used to generate consensus sequence of the full-length genome using Clustal X in BioEdit software v.7.0. Sequences were submitted to GenBank with accession numbers OQ859619- OQ859622. The sequences were compared with those available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed January 6th, 2023) and were used for phylogenetic analyses.

2.4. Phylogenetic analysis

Phylogeny was performed on DCH genome sequences from this study that were aligned with whole genome DCH sequences and whole genomes of two mammalian orthohepadnaviruses retrieved from GenBank (accessed January 6th, 2023). All sequences were aligned using Clustal X in BioEdit software v.7. Phylogeny was estimated by a neighbour-joining algorithm (NJ) and by maximum likelihood (mL) methods with 1000 bootstrap replicates using MEGA v.11 (Kimura, 1980; Felsenstein, 1981; Tamura et al., 2021). For sequences comparison, the percentage of nucleotide similarity of pairwise evolutionary distances of DCH whole genome sequences of this study and all DCH whole genome sequences available from GenBank was calculated using MEGA v.11.

3. Results

3.1. Caseload

In total, 72 cats were included in this study, 33 and 39 cases with and without cavitary effusion respectively. Characteristics of analyzed cats are summarized in Table 2. All serum samples (72/72) were subjected to molecular analysis. For the 33 cats with cavitary effusions, at least one residual effusion sample for PCR analysis was present for 28 (28/33; 84.8%) cats. Information regarding samples of cavitary effusions analyzed by DCH qPCR based on their classification are summarized in Supplementary material (Table S1). The liver biopsy was available for one cat. Follow-up samples collected at the moment of discharge (after 4 months from admission) were present for one cat.

3.2. DCH detection

Overall, DCH was detected in serum samples by qPCR in 2/72 (2.8%; 95% confidence interval: 0–6.6%) of the tested cats. Both DCH-positive cats belonged to the group with cavitary effusions, while all serum samples of the cats without effusion were DCH-negative (Table 2). DCH was also detected in peritoneal effusions of both DCH-positive cats, whereas DCH was not detected in the pleural and pericardial effusions of the DCH-positive cat with multiple effusions (identified as cat no. 1). According to the effusion type, DCH was detected in one inflammatory (cat no. 1) and one non-inflammatory effusion (cat no. 2) (Table 2). The liver biopsy obtained from cat no. 1 was DCH-negative based on immunohistochemical results.

Results of qPCR, including viral quantification of the two DCH-positive cats, are reported in Table 3.

3.3. Characteristics of DCH-positive cats

Both DCH-positive cats were domestic shorthair privately owned cats admitted to the VTH with a history of hyperthermia, diffuse edema, suspected liver disease and cavitary effusions. In both cases cats were

Table 1
PCR primers used to amplify DCH whole genome sequence.

Fragment	Oligonucleotides	Sequence (5′–3′)	Position*	Reference
No.1	HBV-pol-F1	TAGACTSGTGGTGGACTTCTC	95–587	Wang et al., 2017
	HBV-pol-R1	CATATAASTRAAAGCCAYACAG		
No.2	FHBV-for	CGTCATCATGGGCTTTAGGAA	458–1281	Lanave et al., 2019;Aghazadeh et al., 2018
	Cir7-R	CGTAGACGAAGGACACGTC		
No.3	DCH 1127 F	GGAACTCATTGCTGCCTGCGTAG	1127–1885	This study
	DCH 1885R	TCCCTACCTGTAAGTTCCTCTTCAT		
No.4	DCH 1733 F	TAGATGGCTTTGGGGTATGGACAT	1733–2433	This study
	DCH 2433 R	ATCAGCAGGCACATGGATATT		
No.5	DCH 2282 F	TCGCAGACGTTCTCAATCTCCA	2282–3101	This study
	DCH 3101 R	GTCCAGGTGCAGCAGTTGTCTTG		
No.6	DCH 2910 F	GCTCTCTCAGTTTCGCAACCCA	2910–290	This study
	DCH 290 R	AGAACCAACGAGAAGATGAGCA		

* nucleotide position according to Australian reference strain AUS/2016/Sydney (GenBank accession nr. NC_040719).

Table 2
Characteristics of cats analyzed in this study and DCH positivity in serum samples.

Population characteristics		Cats with effusions (no. 33)	Cats without effusions (no. 39)	Total cats (no. 72)	
		No. (%)	No. (%)	No (%)	Serum DCH positive No. (%)
Sex	Male	22 (51.2)	21 (48.8)	43 (59.7)	1 (2.3)
	Female	11 (37.9)	18 (62.1)	29 (40.3)	1 (3.4)
Age[a]	< 2 years	5 (41.7)	7 (58.3)	12 (17.6)	1 (8.3)
	≥ 2 years	26 (46.4)	30 (53.6)	56 (82.4)	1 (1.8)
Breed	Domestic shorthair	30 (46.2)	35 (53.8)	65 (90.3)	2 (3.1)
	Purebreed	3 (42.9)	4 (57.1)	7 (9.7)	-
Ownership	Owned	17 (36.2)	30 (63.8)	47 (65.3)	2 (4.2)
	Stray	11 (64.7)	6 (35.7)	17 (23.6)	-
	Shelter	5 (62.5)	3 (37.5)	8 (11.1)	-
FIV status[b]	FIV positive	5 (55.6)	4 (44.4)	9 (22)[c]	-
	FIV negative	18 (56.3)	14 (43.7)	32 (78)	2 (6.2)
FeLV status[b]	FeLV positive	5 (71.4)	2 (28.6)	7 (17.1)[c]	-
	FeLV negative	18 (52.9)	16 (47.1)	34 (82.9)	2 (5.9)
Biochemical changes potentially consistent with liver disease	No	16 (32)	34 (68)	50 (69.4)	0 (0)
	Yes	17 (77.3)	5 (22.7)	22 (30.6)	2 (9.1)
Likelihood of liver disease	Low	4 (57.1)	3 (42.9)	7 (9.7)	-
	Intermediate	9 (100)	-	9 (12.5)	2 (22.2)
	High	4 (66.7)	2 (33.3)	6 (8.3)	-
Effusion	Present	33 (100)	-	33 (45.8)	2 (6.1)
	Absent	-	39 (100)	39 (54.2)	-
Localization of effusion	Peritoneal only	20 (60.6)	-	20 (27.8)	1 (5)
	Pleural only	7 (21.2)	-	7 (9.7)	-
	Pericardial only	1 (3)	-	1 (1.4)	-
	Peritoneal and pleural	4 (12.1)	-	4 (5.6)	-
	Peritoneal, pleural and pericardial	1 (3)	-	1 (1.4)	1 (100)
Inflammatory vs non inflammatory effusion[d]	Inflammatory	18 (54.5)	-	18 (25)	1 (5.6)
	Non inflammatory	15 (45.5)	-	15 (20.8)	1 (6.7)
Type of effusion[d]	septic	4 (12.1)	-	4 (5.6)	-
	FIP	3 (9.1)	-	3 (4.2)	-
	non specific exudate	11 (33.3)	-	11 (15.3)	1 (9.1)
	neoplastic	6 (18.2)	-	6 (8.3)	-
	hemorrhagic	3 (9.1)	-	3 (4.2)	-
	chylous	1 (3)	-	1 (1.4)	-
	uroperitoneum	1 (3)	-	1 (1.4)	-
	transudate	4 (12.1)	-	4 (5.6)	1 (25)

[a] Age was unknown for four cats.
[b] FIV and FeLV status was unknown for thirty-one cats.
[c] Two cats were positive for both FIV and FeLV.
[d] Cats with more than one effusion were classified according to peritoneal effusion
- negative results (0)

Table 3
Results of DCH quantification in positive samples.

	Serum DCH DNA copies/mL	Peritoneal effusion DCH DNA copies/mL	Pleural effusion DCH DNA copies/mL	Pericardial effusion DCH DNA copies/mL
Cat no. 1	3.3×10^4	2.7×10^4	Negative	Negative
Cat no. 2	1.6×10^9	7.8×10^6	Effusion not present	Effusion not present

FIV-FeLV negative and bacterial culture and reverse transcription qPCR for FCoV RNA in the effusion were negative. Results of the biochemical, hematological and effusion analysis of the two cats are summarized in Table 4.

In cat no. 1 (female, 6 years old), diagnostic imaging revealed the presence of pericardial, pleural and peritoneal effusions along with liver enlargement, with the presence of multiple hyperechoic lesions diffused to all the liver parenchyma. Hematology and clinical chemistry were

Table 4
Hematological and biochemical characteristics of the two DCH-positive cats.

Sample	Hematological and biochemical characteristics (range values)	Cat no.1 at admission	Cat no.1 follow-up	Cat no.2 at admission
Blood and serum	Ht (24–45%)	33.0	40.9	27.7
	RBC (5.7–10 × 10^6 /μL)	8.43	10.44	6.12
	WBC (6.0–17 × 10^3 /μL)	10.68	8.46	6.48
	Total Proteins (5.4–8.5 g/dL)	7.1	8.3	4.2
	Albumin (2.1–3.3 g/dL)	3.2	3.9	1.3
	AST (26–43 IU/L)	54	ND	ND
	ALT (<83 IU/L)	267	114	21 *
	ALP (25–93 IU/L)	69	7	9 *
	GGT (<5 IU/L)	3	1	ND
	Total Bilirubin (<0.5 mg/dL)	0.45	ND	1.74
Peritoneal effusion	Total Proteins (g/dL)	3.5	Effusion not present	0.3
	Cellularity (10^3 /μL)	6.57		0.48
	Main cell populations detected at cytology	Lymphocytes and non-degenerated neutrophils, more rarely foamy macrophages, plasma cells eosinophils, reactive mesothelial cells and mott cells		Foamy macrophages, non-degenerated neutrophils, lymphocytes, more rarely eosinophils and mast cells
	Macroscopic characteristics	Mildly sero-hematic		Transparent, colorless
Pleural effusion	Total Proteins (g/dL)	2.4	Effusion not present	Effusion not present
	Total nucleated cell count (10^3 /μL)	2.38		
	Main cell populations detected at cytology	Lymphocytes and non-degenerated neutrophils, foamy macrophages displaying cytophagy, plasma cells more rarely eosinophils and metarubricyte		
	Macroscopic characteristics	Mildly sero-hematic		
Pericardial effusion	Total Proteins (g/dL)	4.0	Effusion not present	Effusion not present
	Total nucleated cell count (10^3 /μL)	13.5		
	Main cell populations detected at cytology	Non-degenerated neutrophils, foamy macrophages foamy macrophages displaying cytophagy, more rarely lymphocytes, plasma cells eosinophils and reactive mesothelial cells		
	Macroscopic characteristics	Hemorrhagic		

Abbreviations: Ht, hematocrit; RBC, red blood cells; WBC, white blood cells; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; ND, not determined.
* elevated liver enzymes before admission to the VTH.

unremarkable, except for a mild increase of AST and ALT. Therefore, the cat has been included in the intermediate likelihood of liver disease group. Based on the criteria listed in the material and methods section, the peritoneal and pleural effusions were classified as non-specific exudate, whereas pericardial effusion was hemorrhagic. The patient was initially treated with nonsteroidal anti-inflammatory and antibiotic therapy, without any clinical improvement. Consequently, regarding pericardial effusion, an effusive-constrictive pericarditis was supposed and subtotal pericardiectomy was performed by median sternotomy. During the same surgery, a core biopsy of the liver was collected. Intraoperative impression cytology revealed the presence of reactive lymphocytes and plasma cells in the liver and a few inflammatory cells in the pericardium. The cytological features were consistent with lymphoplasmacytic hepatitis, confirmed by histopathology. The histology of the pericardium was consistent with the diagnosis of fibrous pericarditis. The cat clinically improved, including the progressive disappearance of the effusion, over a 3 months period, and was discharged. ALT activity, that was mildly elevated at admission, strongly decreased at discharge, although values were still outside the reference intervals (Table 4). No other significant clinical, hematological and biochemical findings were reported on follow-up clinical examination after four months after the hospitalization (Table 4). Moreover, serum qPCR for DCH was negative during the follow-up of the cat.

In cat no. 2 (male, 1 year old), which had a history of elevated liver enzymes, abdominal ultrasonography revealed the presence of diffuse peritonitis and lymph node enlargement, along with the presence of peritoneal effusion. Hematology was unremarkable, while clinical chemistry revealed some changes potentially consistent with liver failure (hypoalbuminemia, decreased enzyme activity, hyperbilirubinemia), and therefore, also based on the classification of the effusion as a pure transudate, the cat was included in the intermediate likelihood of liver disease group. The cat was treated with nonsteroidal anti-inflammatory drugs, anti-hypertensive drugs and hepatoprotective agents. The cat was discharged upon request of the owners. Seven months after this episode, the owner reported good health conditions for the cat.

3.4. DCH whole genome sequencing and phylogenetic analysis

All six genome fragments were efficiently amplified only from samples with Ct $\leq$ 32 and whole genome sequences of 3184 bp were obtained for serum and peritoneal effusion of cat no. 2. In the presence of Ct $>$ 32, five fragments (no. 1–2 and no. 4–6, corresponding to the partial C, P and X genes and the complete S gene) and two fragments (no. 1 and 6, corresponding to partial P and S genes) were obtained from the serum and the effusion sample of cat no. 1, respectively. Therefore, partial whole genome sequence of about 85.9% (2736 bp) of the genome was obtained from serum sample, whereas a partial sequence of 789 bp

was obtained from the effusion sample of cat no.1.

Both serum and effusion sample of cat no. 2 showed identical whole genome viral sequences (100% nucleotide similarity). Similarly, serum and effusion sequences of cat no. 1 showed the same partial viral sequence. The DCH 789 bp sequence from effusion of cat no. 1 (GenBank accession number OQ859622) was not included in further nucleotide comparison and phylogenetic analyses. Nucleotide pairwise identity between the whole genome sequences of cat no. 2 and the nearly complete DCH genome sequence from serum of cat no. 1 was 99.9% (Table S2). BLAST analysis and nucleotide comparison (Table S2) showed that the closest nucleotide similarity (98.7% and 98.6% for cat no. 2 and cat no. 1, respectively) was to the prototypic Australian sequence (GenBank Accession number NC_040719). The DCH whole and nearly complete genome sequences from the two cats of this study showed nucleotide similarity ranging from 96.8% to 97.4% with other DCH sequences from Italy (GenBank Accession numbers MK117078, OK574325, OK574326 and OM785182). The whole and nearly complete genome DCH sequences from this study showed > 96.0% nucleotide similarity with all the other DCH sequences except for the DCH sequence from isolate Rara, Japan (GenBank accession number LC685967), which showed nucleotide similarity < 92% (Table S2). Moreover, after DNA sequence identity comparison, results showed nucleotide differences > 8% of the DCH sequence from Japan, isolate Rara with all other DCH sequences available in GenBank, indicative of another DCH genotype (putative genotype B) compared to the rest of the sequences (putative genotype A).

The phylogenetic tree based on mL analysis of whole genome sequences (Fig. 1) confirmed that the DCH sequences of this study clustered with the putative genotype A, within the prototype Australian DCH sequence (clade A1) but were not included in the clade A2 with other DCH sequences from Italy (Capozza et al., 2023). NJ analysis showed similar results (data not shown).

4. Discussion

Since the discovery of the novel DCH, different studies have detected the presence of DCH in cats in Asia, Oceania, Europe and America (Aghazadeh et al., 2018; Lanave et al., 2019; Piewbang et al., 2020; Anpuanandam et al., 2021; Stone et al., 2022). Results of our study showing a 2.8% DCH positivity confirm that DCH is circulating in cats in Italy, where DCH infection has been previously reported ranging between 4.2% and 10.8% (Lanave et al., 2019; Scavone et al., 2022). Nevertheless, it should be considered that the DCH infection detected by molecular investigations (Aghazadeh et al., 2018; Lanave et al., 2019; Piewbang et al., 2020; Anpuanandam et al., 2021; Jeanes et al., 2022; Scavone et al., 2022; Stone et al., 2022; Takahashi et al., 2022; Capozza

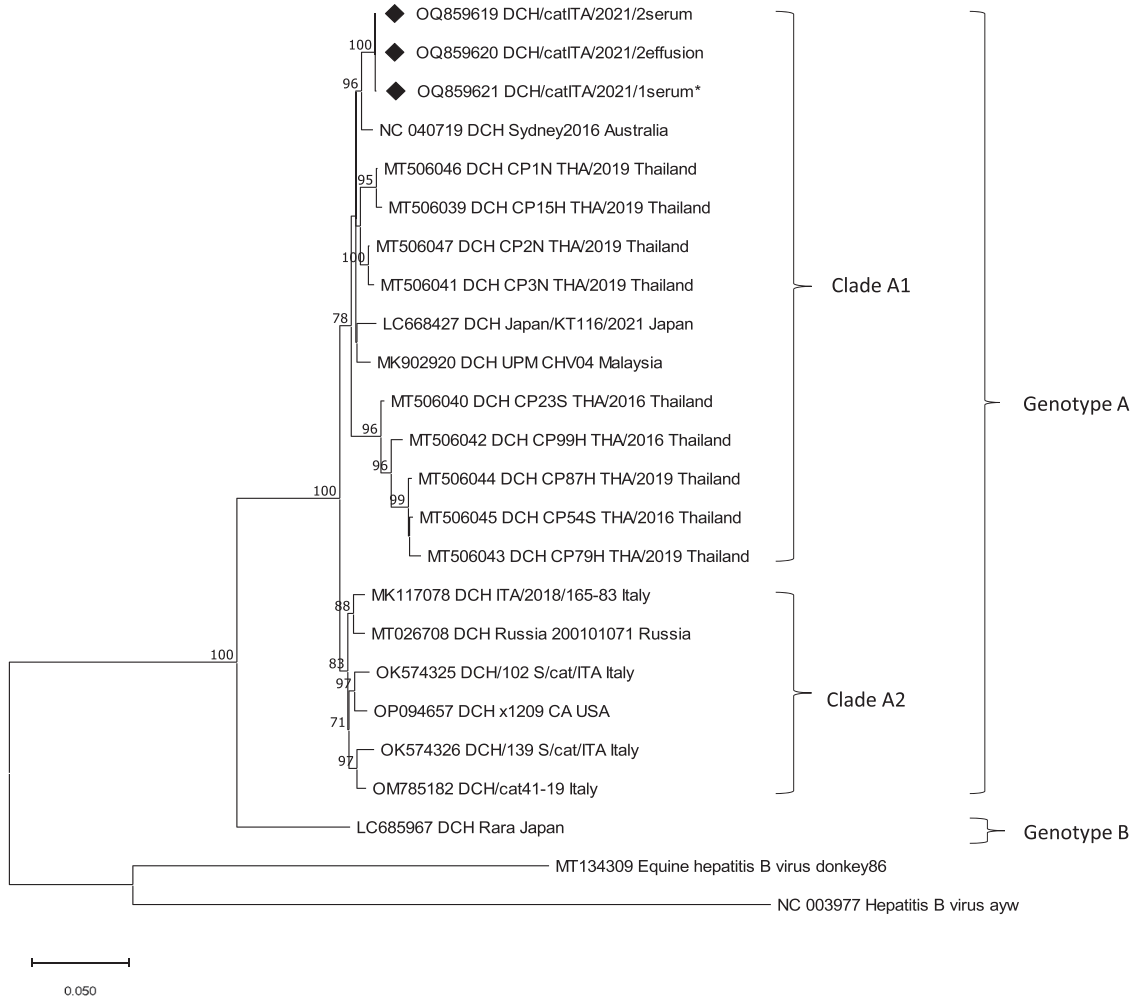

Fig. 1. Phylogenetic tree generated with maximum likelihood analysis. Whole genome sequences from domestic cat hepadnavirus (DCH) retrieved from GenBank databases and the DCH obtained in this study were used. Mammalian orthohepadnaviruses whole genome sequences were used as outgroup. Sequences are indicated by GenBank accession number (available at www.ncbi.nlm.nih.gov/pubmed/) name of the strain and geographical origin. Distances were computed using the General Time Reversible model using MEGA v.11. Bootstrap values above 70% are shown. The black diamonds indicate the sequences obtained in this study; * indicates partial whole genome sequence.

et al., 2023) may be underestimated. Indeed, a high percentage of DCH infected cats has been identified using an optimized serology protocol able to identify anti-DCHc antibodies both with and without DCH DNA detectable at the blood/serum level (Fruci et al., 2022). In this respect, the absence of serological testing in this study has likely limited the identification of DCH-infected cats. Trying to make a parallelism with HBV patterns of infection in humans, DCH infection may also mirror HBV chronic infection that may be characterized by low viremia, normalization of the ALT levels and presence of antibodies against HBV core protein (anti-HBc Abs) whereas the presence of antibodies against HBV surface protein (anti-HBs Abs) is variable (Terrault et al., 2018; Fruci et al., 2022). Therefore, to assess DCH prevalence, diagnostic procedures in cats should profile the stage of infection by detecting antibodies, antigens and viral DNA, as reported for HBV infections in humans. Indeed, some forms of HBV (i.e. occult infections) may be undetected by PCR alone because of low viral loads, in serum samples (Terrault et al., 2018).

DCH whole genome analysis has been used for DCH genetic diversity assessment and molecular epidemiology. Analysis of DCH whole genome may help to clarify pathobiology and define if some viral variants may be associated with different clinical outcomes, as previously reported in humans infected with HBV (Gao et al., 2015). However, it should be considered that whole genome sequencing may be challenging in the presence of low viral loads. Indeed, in this study DCH complete genomes were obtained only for the samples of cat no. 2 showing Ct < 32, corresponding to $> 10^4$ copies/mL of viral DNA that is usually required for whole genome sequencing (Margeridon et al., 2008). In the two samples from cat no.1, which showed lower viral loads compared to cat no.2, the discontinuous structure of relaxed circular (RC)-DNA of hepadnaviruses may have also accounted for the problematical amplification of at least one of the fragments (Margeridon et al., 2008). In this regard, rolling circle amplification or next-generation sequencing technique may be suggested to perform DCH whole genome sequencing analysis in samples with low viral load, as previously reported for other hepadnaviruses (Margeridon et al., 2008; Diakoudi et al., 2022). Moreover, cell culture systems for DCH isolation are also warranted.

Phylogenetic analysis based on whole genome sequences showed that the DCH sequences detected in this study were not included in the clade with other Italian DCH strains, one of which was recently reported from the same study area, namely the Lombardy region (Scavone et al., 2022). The presence of two different DCH clades (A1 and A2), clustering Italian sequences and belonging to one DCH genotype in Italy, was highlighted. This result further confirms genetic diversity of DCH within the same geographic area (Piewbang et al., 2020; Stone et al., 2022).

The potential pathogenetic role of DCH in liver disease is currently under investigation in cats (Lanave et al., 2019; Pesavento et al., 2019; Anpuanandam et al., 2021; Capozza et al., 2021a; Piewbang et al., 2022). Our study showed that the two cats with $\geq 10^4$ DCH DNA copies/mL in serum were likely affected by liver damage, confirming the positive correlation of DCH viral copy numbers with ALT values and with the degree of hepatitis in cats (Piewbang et al., 2022). The same results have been reported in HBV-infected humans for which 10^4 HBV DNA copies/mL in serum samples have been considered as the lower threshold for hepatitis and liver damage (Seeger et al., 2013). Despite the increased liver enzymes in biochemical analyses prior to VTH admission, in our study the likelihood of liver disease of cat no. 2 was based on the increase of total bilirubin (not associated with hematological changes consistent with hemolysis or with changes suggestive of cholestasis) coupled with hypoalbuminemia. Indeed, it may be postulated that also this cat experienced some type of transient liver damage and that low ALT activity observed during admission could be due to the short half-life of this liver enzyme in cats, coupled with the possible occurrence of an ongoing liver failure supported also by hypoalbuminemia and a pure transudate (Hoffmann et al., 1977; Stockham and Scott, 2008). Therefore, we suggest that the likelihood of liver disease should rely not only on biochemical markers of liver damage, such as ALT and ALP that may be within reference interval and cannot rule out chronic hepatitis in cats (mirroring normal biochemistry in chronic hepatitis which accompany HBV infection in humans) (Seeger et al., 2013), but also on liver functionality markers, such as high total bilirubin, hypoalbuminemia or, better, bile acids (Stockham and Scott, 2008), that unfortunately have not been tested in the current case. The presence of DCH in cats showing an intermediate but not high likelihood of liver disease confirms previous findings (Scavone et al., 2022), suggesting that DCH infection is likely not correlated with liver disease severity. The ALT decreases during discharge in cat no.1 may indicate that, assuming that a hepatocellular damage was present at admission, a progressive recovery occurred over time. The presence of lymphoplasmacytic hepatitis has already been associated with DCH infection (Pesavento et al., 2019; Piewbang et al., 2022) but unfortunately the lack of DCH DNA in the liver biopsy of cat no. 1 did not allow to define the association of DCH with histopathological features of the liver. Further investigations are needed, considering that the negative PCR results may have been due to the patchy distribution of DCH in the liver, as previously reported (Pesavento et al., 2019).

The results from our study showing that DCH may be present in the peritoneal effusion of DCH-positive cats are not surprising and are in accordance with previous reports on HBV in humans showing the presence of HBV surface antigen (HBsAg) in human ascitic fluids of HBsAg carriers (Salo et al., 1980; Komatsu et al., 2016). The presence of DCH in the effusions of two cats suggests that the hepadnaviral infection may be linked to a cavitary effusion in individual cases. The presence of DCH in the inflammatory peritoneal effusion of cat no. 1 suggests that blood contamination of the peritoneal effusion may be accounted as a possible cause of DCH presence within the effusion although gross and cytological evaluation revealed only minimal blood contamination. On the other hand, the presence of DCH in the non-inflammatory peritoneal effusion of cat no. 2 may be due to altered oncotic pressure resulting from hypoalbuminemia, which is the primary cause of protein-poor transudative effusions in veterinary patients (Dempsey & Ewing, 2011). Results suggests that DCH DNA may be detected in an effusion from a cat that was viraemic at the time of sampling. The presence of DCH only in the peritoneal effusion of cat no.1, while pericardial and pleural effusions were negative, is in accordance with a previous study showing a higher DCH viral load in abdominal organs, compared to lungs or heart (Piewbang et al., 2020). Further investigations are needed to understand the mechanisms that lead to DCH presence in feline cavitary effusions and to define the presence of DCH in other body fluids, as HBV has been previously reported in a range of body fluids from HBV-infected humans (Komatsu et al., 2016).

Overall, results of our study do not suggest that hepadnaviral infections are a differential diagnosis for effusions. Further investigations on DCH pathogenic role are recommended given that a previous study has detected DCH presence in various organs with inflammatory lesions, suggesting a broad cell tropism of DCH and a potential pathogenetic role of DCH in systemic disease (Piewbang et al., 2020; Shofa et al., 2022).

Regarding risk factors, despite the low number of DCH-positive cats did not allow statistical analysis, the detection of DCH in two owned cats is in accordance with previous results showing a significantly higher prevalence in pets compared to shelter cats (Anpuanandam et al., 2021). The reasons for this difference are unclear (Shofa et al., 2022) and could rely on DCH transmission routes which are still unknown (Capozza et al., 2021b). Our results also confirm that DCH-positive cats may be FIV- and FeLV-negative, as previously reported (Stone et al., 2022).

To date, clinical signs of DCH-positive cats have not been clearly defined and healthy DCH-positive cats have been reported (Anpuanandam et al., 2021). Non-specific signs, such as lethargy and anorexia, as well as acute hepatopathy (Anpuanandam et al., 2021; Capozza et al., 2021b), have been observed in DCH-positive cats, mirroring HBV-infected humans that may be asymptomatic or unhealthy according to the progression of the disease (Liang, 2009; WHO, 2022). Unfortunately, it was not possible to confirm if the clinical status observed

in the two DCH-positive cats was due to DCH infection because other pathological conditions that may have caused the clinical status were not investigated, except for FIV, FeLV, FIP and sepsis that were ruled out based on laboratory findings and the clinical outcome (Fischer et al., 2011).

This study has some limitations, starting from the sample size and the identification of infected cats by detection of DCH DNA only and not by inclusion of serology, given that the absence of DNA does not indicate that a cat is uninfected. Therefore, the study design likely underestimated DCH positive cats and did not allow to perform statistical analysis of any association between DCH infection and a clinical outcome. Other limitations are due to the retrospective analysis of samples collected from cats admitted to a VTH, not allowing the availability of all the required samples. In this respect, the absence of follow-up and liver biopsy in cat no. 2 precluded crucial information about the presence of lesions in the liver and its functionality and neither allowed us to confirm the likely ongoing liver insufficiency in cat no. 2 based on clinical chemistry, possibly caused, among other pathophysiological conditions, by chronic hepatitis.

5. Conclusion

In conclusion, to the best of our knowledge, this is the first study that has detected DCH DNA in peritoneal effusions from DCH-positive cats, likely mirroring the presence of HBV in body fluids of HBV-infected humans. Further investigations are still needed to confirm the pathogenic role of DCH in cats.

Ethical approval

Samples were collected according to the diagnostic procedures and according to the Ethical Committee decision of the University of Milan, residual aliquots of samples or tissues collected for diagnostic purposes at the VTH under informed consent of the owners can be used for research purposes without any additional formal request of authorization (EC decision 29 Oct 2012, renewed with the protocol no. 02–2016).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109828.

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