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ORIGINAL RESEARCH

Evaluating the presence of domestic cat hepadnavirus viraemia in cats with biochemical alterations suggestive of liver disease

Donatella Scavone | Stefania Lauzi | Angelica Stranieri | Giada Tramontano | Gabriele Ratti | Saverio Paltrinieri

Department of Veterinary Medicine and Animal Sciences, University of Milan, Lodi, Italy

Correspondence
Stefania Lauzi, Department of Veterinary Medicine and Animal Sciences, University of Milan, Via dell'Università 6, 26900 Lodi, Italy.
Email: stefania.lauzi@unimi.it

Abstract

Background: The association between domestic cat hepadnavirus (DCH) infection and feline chronic hepatitis and hepatocellular carcinoma has been suggested. However, studies focused on the association between DCH infection and clinicopathological changes consistent with liver disease in cats are not available.

Methods: This retrospective investigation included sera obtained from 96 cats that had the serum activity of at least alanine aminotransferase or alkaline phosphatase measured during initial diagnostic work-up. Based on these haematobiochemical results, cats were categorised according to their likelihood of having liver disease (absent, low, intermediate or high). DCH DNA was detected using real-time PCR, nested PCR and sequencing.

Results: Overall, potential liver damage was observed in 44 cats, including cats with low (n = 14), intermediate (n = 10) and high (n = 20) likelihood of liver disease. Four cats (4.2%) were DCH-positive, with three positive cats belonging to the liver disease group (two with low and one with intermediate likelihood of liver disease).

Conclusions: Although the pathogenic potential of DCH in cats still has to be clarified, these results suggest that DCH testing should not be based only on the presence of biochemical changes potentially consistent with liver disease.

KEYWORDS
emerging viruses, feline, hepatitis virus, liver disease

INTRODUCTION

Viruses of the genus *Orthohepadnavirus*, family Hepadnaviridae, infect a variety of mammals including primates, bats and rodents.[1] Hepadnaviruses are typically hepatotropic, and the type species, human hepatitis B virus (HBV), is a major public health problem as chronic infections increase the risk of liver diseases such as cirrhosis and hepatocellular carcinoma.[2–4]

The domestic cat hepadnavirus (DCH) was first discovered in a domestic cat from Australia with multicentric high-grade large B-cell lymphoma and feline immunodeficiency virus (FIV) infection.[5] Since its discovery, some authors have investigated the prevalence of DCH infection, its relationship with retroviral infection and its pathogenic potential related to liver disease.[5–9] In particular, DCH infection was found to be associated with some cases of chronic hepatitis and hepatocellular carcinoma in cats, with histological features mirroring those reported for human HBV-related disease.[8]

The pathogenic potential of DCH in the development of clinical or subclinical liver disease still has to be clarified. In this regard, Lanave et al.[6] reported that almost half of DCH-positive cats for which biochemistry was available (20/42) showed increase in liver structural or functional damage marker, such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and total bilirubin. Similarly, Anpuanandam et al.[9] reported that almost half of DCH-infected cats (12/23) had elevated

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ALT levels, and that cats with abnormal ALT had 21% chance of being DCH infected and viraemic.

However, studies specifically focused on the prevalence of DCH viraemia in cats with clinicopathological changes consistent with liver disease are not available. It is still unknown if it is reasonable to suspect and investigate DCH infection in the veterinary clinical practice when biochemical abnormalities suggestive of hepatocellular damage are present in cats. Therefore, the aim of this work was to define, by a retrospective investigation, if the prevalence of DCH infection is higher in cats with biochemical alterations suggestive of liver disease compared with cats without abnormalities consistent with structural or functional liver damage.

MATERIAL AND METHODS

Animals

The study was performed on cats admitted to the Veterinary Teaching Hospital (VTH) of Lodi between January and December 2019. Serum specimens stored at −20°C were retrospectively selected from the routine diagnostic archive of samples previously sent to the laboratory of clinical pathology for diagnostic purposes. Blood samples collected in tubes with clot activator and submitted to the laboratory had been immediately centrifuged and the biochemical analyses required had been carried out on serum. Residual volumes of serum had been stored at −20°C within the day. According to the guidelines of our institution, a formal approval was not required because leftover serum used in this study was harvested from blood samples collected for diagnostic purposes or as part of health checks and with informed consent of the owners (decision no. 2/2016 of the Ethical Committee of the University of Milan).

Cats and respective samples were included if at least the activity of ALT and ALP had been measured. When other parameters such as AST, GGT, creatine kinase (CK) activity and total bilirubin concentration had been measured, results were considered and recorded as well. Biochemistry analyses had been performed with an automated chemistry analyser (BT 3500, Biotecnica Instruments, Rome, Italy) and reagents provided by the instrument's manufacturer. Data regarding signalment and, when available, clinical history were recorded for each cat. The cats were then categorised into two groups according to their age: ≤2 and >2 years.

Cats were also categorised according to clinical information and biochemical analyses results for liver disease status. Given that the availability of clinical information was not exhaustive, a definite diagnosis of hepatopathy could not always be confirmed or ruled out for all cats. Therefore, cats were classified based on their probability of having liver disease according to biochemical analyses reported above (ALT, ALP and, if present, AST, GGT, CK activity and total bilirubin concentration). In particular, we selected three categories of liver disease probability based on signalment (particularly age) and biochemical results, considering the specificity of each biochemical change for primary liver disease and the magnitude of increase with respect to the relevant upper reference range.[10] The three categories were the following:

- low: less than two-fold increase in ALT activity or more than two-fold increase in ALT activity paired with simultaneous increase in CK activity and normal ALP, GGT activity and bilirubin concentration, suggestive of muscle damage; increase in ALP activity presumably due to isoenzymes of non-hepatic origin, such as increase in bone ALP activity related to growth or concurrent diseases (e.g. hyperthyroidism);[10–12]
- intermediate: more than two-fold and less than five-fold increase in ALT activity; less than two-fold simultaneous increase in ALT and ALP activity presumably not due to extrahepatic causes or not associated with abnormalities in other parameters (e.g. GGT), when available;
- high: more than two-fold simultaneous increase in ALT and ALP activity or more than five-fold increase in ALT activity alone.

The algorithm used to select and classify the samples is reported in Figure 1.

PCR and phylogenetic analysis

Time of storage (ie, time from collection of sample to PCR analysis) was first calculated. DNA was extracted from frozen-thawed serum samples using a commercial DNA extraction kit (NucleoSpin Blood kit, Macherey-Nagle, Germany), following the manufacturer's instructions. DNA pre-analytical quality control targeting vertebrate 12S rRNA locus was performed on randomly selected samples (results not shown).[13]

All samples were subjected to quantitative PCR (qPCR) for specific DCH amplification.[6] Threshold cycle (Ct) number was used as the measure of viral load (the lower the Ct level the greater the amount of target nucleic acid is present in the sample). A pan-hepadnaviral nested PCR was also performed on all samples based on published methods using degenerate primers targeting a 258 bp fragment of the conserved domain of the polymerase gene of viruses in the family Hepadnaviridae in the second reaction.[14] Nested PCR products were visualised under a UV transilluminator on a 1.5% agarose gel stained with ethidium bromide. DCH DNA extracted from a naturally infected cat was used as positive control. A blank control (DNAse-free water sample) was also included in the qPCR and nested PCR reactions every 10 reactions, both at primary and secondary amplification in the nested PCR reaction.

The amplicon of the expected size obtained from the second reaction of the pan-hepadnaviral PCR was purified from positive samples and sequenced using the outer forward and reverse primers used for DNA

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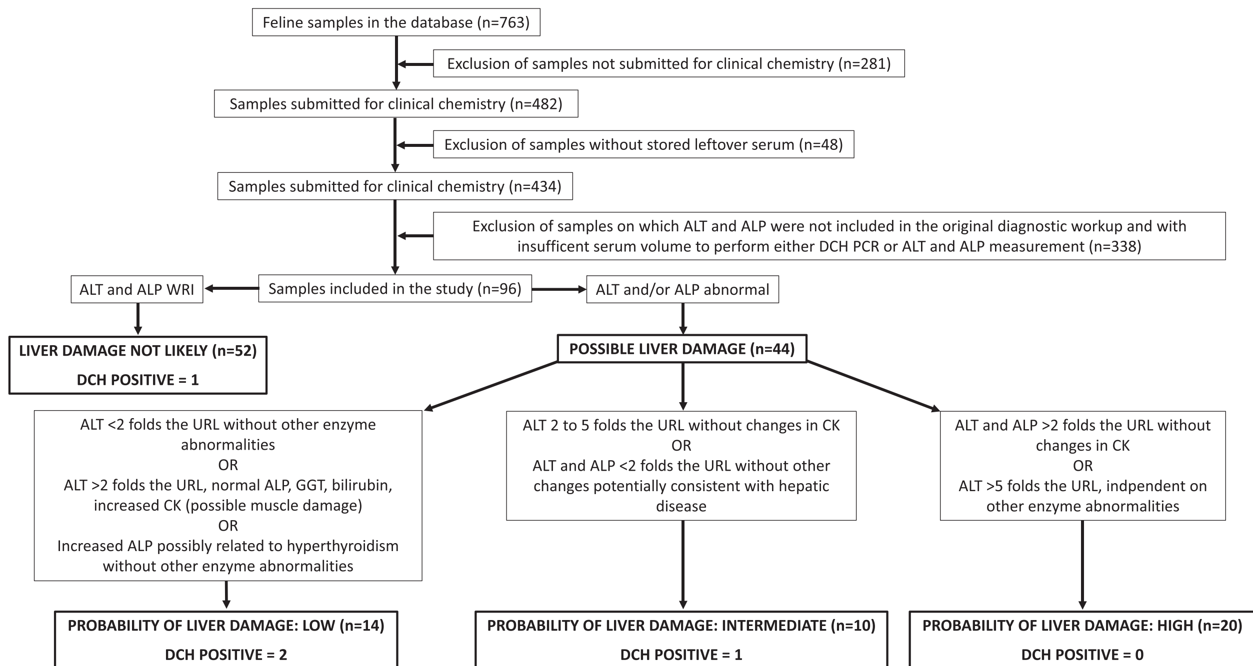

FIGURE 1 Flowchart summarising the criteria and procedure for sample selection. ALP, alkaline phosphatase; ALT, alanine transaminase; CK, creatine kinase; DCH, domestic cat hepadnavirus; GGT, gamma-glutamyl transferase; URL, uniform resource locator; WRI, World Resources Institute

amplification. Sanger sequencing was performed by a commercial sequencing facility (Microsynth Seqlab, Germany). The sequences were then compared with those available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For phylogenetic analysis, the sequences were aligned with DCH reference sequences and with representative sequences of other mammalian orthohepdnaviruses retrieved from GenBank using Clustal X in BioEdit software v.7.0. Phylogeny was estimated by a neighbour-joining algorithm (NJ) and by maximum likelihood methods with 1000 bootstrap replicates using MEGA v.7.[15–17] For sequences comparison, the percentage of nucleotide similarity of pairwise evolutionary distances was calculated using MEGA v.7.

Animals showing positive qPCR and nested PCR results confirmed by sequencing and phylogeny were considered DCH positive. The DCH sequences obtained in this study were deposited in GenBank under accession numbers OM785180-OM785183.

Data analyses

The 95% confidence limits were calculated for DCH-positive cats. Pearson's chi-square test was used to evaluate the differences between proportions of DCH-infected cats and sex, age, type of ownership and likelihood of liver disease status. Cats with missing data on sex or age were not included in the sex and age categories, respectively, and were not analysed. Statistical comparisons were carried out using Epitools, taking $p < 0.05$ as significant.[18]

RESULTS

Caseload and occurrence of biochemical abnormalities

The results of the selection and classification procedures are summarised in Figure 1. Overall 598 cats were admitted to the VTH in 2019 for diagnostic purposes, corresponding to 763 feline samples submitted to the laboratory of clinical pathology. Out of the 482 feline serum samples, 434 residual samples were stored at −20°C, corresponding to samples collected from 301 cats. Among the stored samples corresponding to single sampling from cats with adequate aliquot for PCR analysis, a total of 96 serum samples had ALT and ALP measurements and were included and analysed in this study. Sex and age were unknown for eight and 20 cats, respectively. Among cats with recorded sex and age, 39 were male and 49 were female, with median age of 8 years, ranging from 2 months to 20 years.

Fifty-two cats (54.2%) had no biochemical changes potentially consistent with liver disease, while 44 (45.8%) showed abnormalities in at least ALT or ALP activity and possibly also in other parameters like AST, GGT, CK and bilirubin. Based on the subdivision into categories of likelihood to have liver disease, 14 cats were categorised as low probability, 10 as intermediate probability and 20 as high probability of liver disease. Data regarding signalment and biochemical analyses of these cats are summarised in Table 1 and in extended form in Table S1.

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TABLE 1 Cats' characteristics according to their likelihood of liver disease (number of cats and, between parentheses, percentages)

Variable	Category	Total	Biochemical changes		Likelihood of liver disease		
			No	Yes	Low	Intermediate	High
Age	≤2 years	16	4 (25)	12 (75)	6 (37.5)	1 (6.3)	5 (31.3)
	>2 years	60	38 (63.3)	22 (36.7)	5 (8.3)	5 (8.3)	12 (20)
	nd	20	10 (50)	10 (50)	3 (30)	4 (40)	3 (30)
Sex	Male	39	21 (53.8)	18 (46.2)	6 (15.4)	3 (7.7)	9 (23.1)
	Female	49	26 (53.1)	23 (46.9)	8 (16.3)	7 (14.3)	8 (16.3)
	nd	8	5 (62.5)	3 (37.5)	–	–	3 (37.5)
Purebred cat	No	84	46 (54.8)	38 (45.2)	12 (14.3)	9 (10.7)	17 (20.2)
	Yes	9	4 (44.4)	5 (55.5)	2 (22.2)	1 (11.1)	2 (22.2)
	nd	3	2 (66.7)	1 (33.3)	–	–	1 (33.3)
Type of ownership	Owned	56	35 (62.5)	21 (37.5)	6 (10.7)	4 (7.1)	11 (19.6)
	Stray	40	17 (42.5)	23 (57.5)	8 (20)	6 (15)	9 (22.5)
Total		96	52 (54.2)	44 (45.8)	14 (14.6)	10 (10.4)	20 (20.8)

Abbreviation: nd, not determined.

TABLE 2 Number and percentage of cats with domestic cat hepadnavirus (DCH) according to characteristics of cats and likelihood of liver disease

Variable	Category	No. tested	DCH (%)
Age[a]	≤2 years	16	2 (12.5)[b]
	>2 years	60	1 (1.7)[b]
Sex	Male	39	2 (5.1)[b,c]
	Female	49	2 (4.1)[b]
Purebred cat	No	84	4 (4.8)[b,c]
	Yes	9	0 (0)
Type of ownership	Owned	56	1 (1.8)[b]
	Stray	40	3 (7.5)[b,c]
Biochemical changes potentially consistent with liver disease	No	52	1 (1.9)[b]
	Yes	44	3 (6.8)[b,c]
Likelihood of liver disease	Low	14	2 (14.3%)[b]
	Intermediate	10	1 (10.0%)[c]
	High	20	0 (0.0)

[a]Age was unknown for one DCH-positive cat.
[b]DCH-positive cats with threshold cycle (Ct) value >38.
[c]DCH-positive cats with Ct value of 32.1.

Occurrence of DCH and phylogeny

Serum samples were stored at −20°C for a median of 255 days (ranging from 14 to 349 days) before performing DCH analysis (Table S1). Overall, four cats tested positive using both the nested PCR and the qPCR reactions, with one positive sample showing a Ct value of 32.1 and the other three positive samples showing very high Ct values (Ct > 38) (Tables 2 and S1). Storage of the four positive samples ranged from 140 to 267 days (Table S1). The four sequences that were amplified using the pan-hepadnaviral PCR were further confirmed as DCH by BLAST analysis that showed the highest nucleotide similarity ranging from 96.1% to 99.2% with DCH sequences. The nucleotide percentages of similarity of pairwise evolutionary distances among the sequences detected in this study was 97.6%–99.2%. The phylogenetic tree based on maximum likelihood analyses showed that all the four sequences of this study clustered with DCH sequences and not with other orthohepadnaviruses (Figure 2). Results of the phylogenetic analysis were similar using the NJ method (data not shown). Our results showed a 4.2% (95% confidence interval: 0.2–8.2) DCH prevalence in the whole population of tested cats.

Factors associated with DCH positivity

Three of the four DCH PCR-positive cats showed abnormalities in at least ALT and ALP and, according to the criteria used in our study, the prevalence of positive cats among patients with increased ALT and/or ALP accounts for 6.8%. However, none of the positive cats had a high probability of liver disease based on the results of serum enzymes, since these three DCH-positive cats were categorised as having low ($n = 2$) and intermediate ($n = 1$) likelihood of liver disease.

The statistical analyses showed no significant association of DCH positivity with sex, age, type of ownership or liver disease status (Table 2).

DISCUSSION

We evaluated the frequency of DCH infection in the blood of cats with and without alterations in serum biomarkers suggestive of liver disease. The overall prevalence of DCH viraemia obtained in this study (4.2%) is in accordance with previous studies showing prevalence ranging from 1.9% to 12.4%, with differences in the sampled populations possibly due to geographical variances and control measures.[5–7,9,19] Further investigations are needed to assess if the geographical areas with different endemicity and different transmission dynamics previously reported for HBV may also account for DCH infection in cats.[20]

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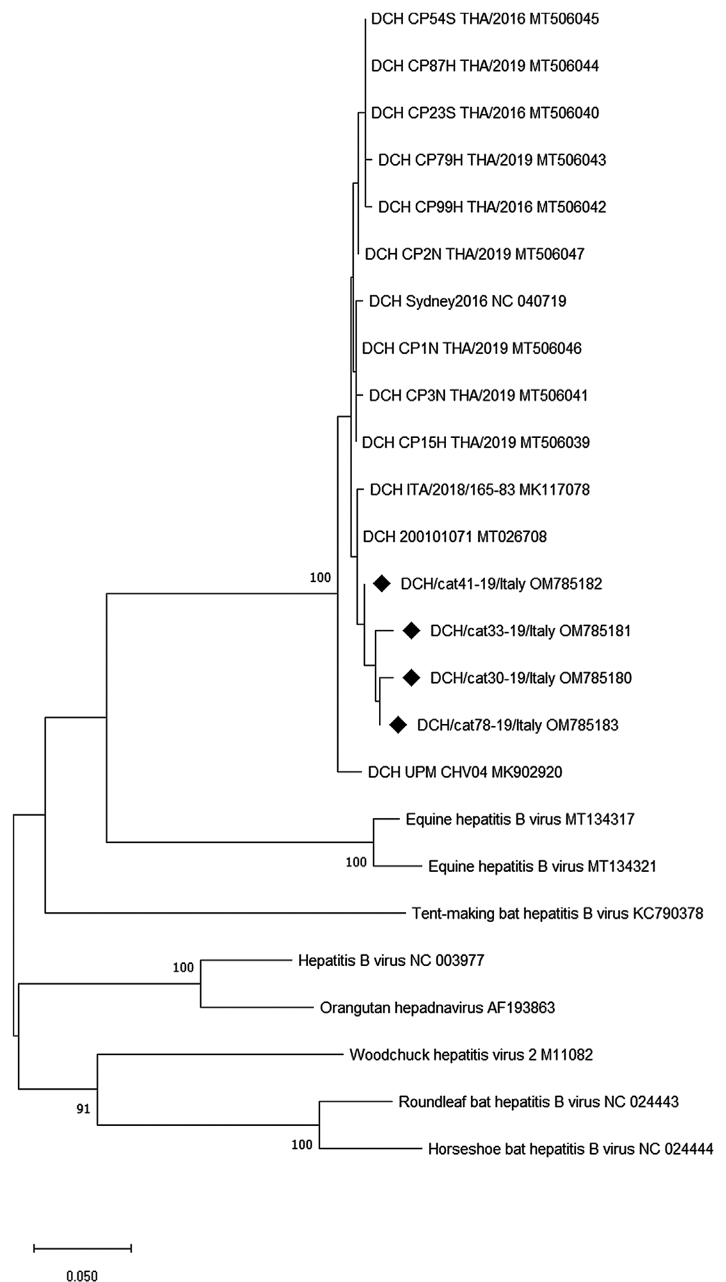

FIGURE 2 Phylogenetic tree generated with maximum likelihood analysis. A 258 bp region of the polymerase gene obtained from mammalian *Orthohepadnavirus* sequences retrieved from GenBank databases and the domestic cat hepadnavirus (DCH) representative sequence obtained in this study was used. Sequences are indicated by GenBank accession number (available at www.ncbi.nlm.nih.gov/pubmed/) and name of the strain. Distances were computed using the General Time Reversible model using MEGA v.7. Bootstrap values above 70% are given. The black diamonds indicate the sequences obtained in this study

Differences in the efficiency of DNA extraction based on the use of different commercial kits and storage of samples may have accounted for the prevalence of DCH infection recorded in this study. DCH DNA in serum samples stored at −20°C may be damaged, and therefore low/intermediate viral loads may go undetected. However, positive samples were also found among those collected at the beginning of the study period, suggesting that long storage may not be the only issue accounting for the low prevalence of infection. In order to confirm DCH prevalence, diagnostic procedures in cats should include a panel of antibodies and antigens and viral DNA to profile the stage of infection, as reported in humans since there are some forms of HBV (i.e. occult infections) in which viral DNA is present at low titer, or it is not detected at all, in serum samples.[21]

The kinetic of DCH in serum as well as DCH transmission dynamics in cats are still unknown. Long-term infection, with DCH detectable in serum, has been recently reported in a proportion of DCH-infected cats.[22] Transmission occurring through blood and body fluids, sexual intercourse and transplacental route, as reported for HBV in humans and for FIV and feline leukaemia virus (FeLV) in cats, could be hypothesised for DCH as well.[6,23,24] In this regard, close contact with other cats may pose a risk for DCH infection. Therefore, stray and free-roaming cats, and also client-owned cats with an outdoor lifestyle, may have a higher risk of infection from DCH, as happens for retroviral infections.[25,26] It is interesting to observe that, among the DCH-positive cats found in our study, the majority of cats were housed in shelters or colonies, whereas only one was a client-owned cat. However, further investigations are needed to define type of ownership of cats as a risk factor for DCH infection because significant differences between stray cats and privately owned cats were not detected in our study. Moreover, the only other study that investigated this aspect detected a significantly higher prevalence

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of DCH in pet cats compared to shelter cats.[9] Our results showing that sex and age are not associated with DCH infection are confirmed by recent reports.[19] However, definition of age as a risk factor probably needs further investigations because both a significant association of DCH infection in cats older than 2 years as well as higher odds of being DCH positive in cats aged 4–7 months, albeit not significantly, have been previously reported.[6,9]

The association between hepadnaviral and retroviral infection previously reported in cats, mirroring HBV and HIV infections in humans, was not investigated in our study due to the lack of information regarding FIV and FeLV status of cats.[5,6,9,27] These data were available only for two DCH-positive cats and one of them was FIV infected. Unfortunately, residual volume of other samples was not enough to perform viral antigen and antibody detection.

Regarding the pathogenic role of DCH, abnormalities in ALT or ALP activity and often in other possible markers of liver damage or dysfunction were detected in three out of four DCH-positive cats. This may suggest an association between DCH infection and liver disease. However, it should be considered that statistical analysis, despite potentially affected by the low number of observations, did not reveal a significant difference between the 3/44 (6.8%) DCH-positive cats included in this study with biochemical abnormalities potentially consistent with liver diseases (increased ALT or ALP, and in most cases other possible biomarker of hepatic dysfunction of damage) and the 1/52 (1.9%) positive cat belonging to the group without abnormalities, suggesting that DCH viraemia does not predict liver disease. The proportion of positive cats with enzyme abnormalities is lower than that reported in a recent study, on which, however, the threshold to classify as 'high' the ALT activity was so close to the upper reference limit to likely include mostly cats without hepatic diseases.[9] As a matter of fact, in the absence of information on the actual presence of liver disease based on clinical findings, diagnostic imaging or histopathology, that is a limitation of this and of many other retrospective studies, we estimated the likelihood of liver diseases rather than presence/absence of liver damage. The categorisation of likelihood of liver disease was based on the cross-matching of the presence of biochemical changes in serum, with special emphasis on liver enzymes and paying attention either on the magnitude of the increase of single enzymes, or on the simultaneous change of more than one enzyme and the information about signalment of the cats. From this standpoint, only very high values of ALT activity alone (>5-fold increase) were considered as highly suggestive of liver damage, in accordance with recent recommendations in cats, although in people the magnitude of increases potentially consistent with liver damage is even higher.[28,29] Moreover, if ALT and/or ALP increase were moderate, we suspected liver disease in the presence of simultaneous increase of other enzymes potentially related with liver damage, since slight or mild increase of ALT and/or ALP alone may depend on extrahepatic causes.[28,30] Unfortunately, the lack of additional information did not allow us to definitely conclude which type of liver disease, if any, was actually present and therefore it was not possible to compare the results of DCH testing with some specific type of liver disease observed in cats that usually induces relevant changes in liver enzymes such as lymphocytic cholangitis.[31] Conversely, we were aware that the lack of changes in ALT and/or ALP may have not excluded the presence of all hepatopathies, since their activity may remain within reference intervals if the functional hepatic mass is reduced (e.g. hepatic chirrosis) or when dysfunctional hepatocytes are not structurally damaged (liver failure or insufficiency).[30] Our analysis revealed that despite the increase of ALT and ALP, more than half of the cats with biochemical signs potentially consistent with hepathopathies (i.e. the cats with low or intermediate probability of hepatopathy), were likely not affected by hepatobiliary diseases and a high probability of liver disease was detected in only a quarter of the total cats analysed. Interestingly, none of the cats with high probability of liver disease was DCH positive suggesting that the possible association between DCH infection and liver disease hypothesised only on the basis of increased ALT or ALP is not supported by the individual analysis of biochemical and signalment data of the cats included in the study. In humans, high viral load in HBV infection has been correlated with virus replication and hepatocyte damage, and a similar correlation has been suggested for DCH in cats.[6] In this respect, our results showing Ct values indicative of higher viral load in the sample from the cat with intermediate probability of liver disease compared to the lower viral loads in the samples of cats without biochemical abnormalities and with low probability of liver disease support this hypothesis but further investigations are needed. The presence of a correlation between DCH infection and liver disease still needs to be established, as recently suggested.[22]

However, it should be considered that the prevalence of DCH in blood samples is lower than in liver tissue samples, since in some stages of the infection DCH DNA is only detectable in liver tissue.[9] Consequently, it is important to outline that the prevalence of viraemia does not reflect, but rather underestimates, the true prevalence of DCH infection. Therefore, a more detailed information about the possible association between a clinical diagnosis of liver disease and DCH infection should be based on PCR and histopathological analysis of liver biopsies that, however, are not routinely collected in routine practice.

Overall, the limitations of our study include possible storage artefacts, poor knowledge of the biology of the virus and kinetic of DCH infection that would help to understand the impact of DCH on the feline population, absence of liver biopsies that does not allow to definitely conclude that cats with increased enzyme activity actually have liver disease and/or to define which type of liver disease is present

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and absence of whole genome sequences of the viruses.

In summary, our study showed that only a small proportion of cats with biochemical alterations suggestive of liver disease were DCH positive, and that DCH was not detected in cats that, based on clinical chemistry, had a high probability of liver disease. This suggests that further testing for DCH (i.e. liver biopsies, PCR on blood or hepatic tissues) should not be based only on the presence of biochemical changes potentially consistent with liver disease.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this study (financial or personal).

FUNDING INFORMATION

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ETHICS STATEMENT

Samples used in this study were retrospectively selected from the routine diagnostic archive of samples previously sent to the Laboratory of Clinical Pathology of the Veterinary Teaching Hospital of Lodi, Italy for diagnostic purposes. According to the guidelines of our institution, a formal approval was not required because leftover serum used in this study was harvested from blood samples collected for diagnostic purposes or as part of health checks and with informed consent of the owners (decision no. 2/2016 of the Ethical Committee of the University of Milan).

AUTHOR CONTRIBUTIONS

Conceptualisation, investigation, writing—original draft: Donatella Scavone. Methodology, writing—original draft: Stefania Lauzi. Investigation, writing—review and editing: Angelica Stranieri, Giada Tramontano and Gabriele Ratti. Conceptualisation, supervision, writing—review and editing: Saverio Paltrinieri.

DATA AVAILABILITY STATEMENT

Data that support the finding of this study area available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Saverio Paltrinieri https://orcid.org/0000-0001-7117-7987

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