A longitudinal observational study in two cats naturally-infected with hepadnavirus.

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Abstract (268)

Hepatitis B virus (HBV) is a major cause of liver disease in humans including chronic hepatitis and hepatocellular carcinoma. Domestic cat hepadnavirus (DCH), a novel HBV-like hepadnavirus, was identified in domestic cats in 2018. From 6.5% to 10.8% of pet cats are viremic for DCH and altered serological markers suggestive of liver damage have been identified in 50% of DCH-infected cats. DCH DNA has been detected in association with characteristic lesions of chronic hepatitis and with hepatocellular carcinoma in cats, suggesting a possible association. In this study longitudinal molecular screening of cats infected with DCH was performed to determine if DCH can cause chronic infections in cats. Upon re-testing of sera from five DCH-positive animals, 2-10 months after the initial diagnosis, three cats tested negative for DCH on two consecutive occasions using quantitative PCR. Two other cats remained DCH-positive, including an 8-month-old female cat re-tested four months after the initial positive result, and a 9-year-old male cat, which tested positive for DCH on six occasions over an 11-month period. The latter had a history of chronic hepatopathy with jaundice, lethargy and elevated serum alanine transaminase levels (ALT). During the period of observation, DCH titers ranged between $1.64x10^5$ and $2.09x10^6$ DNA copies/mL and ALT was persistently elevated, suggesting chronic infection. DCH DNA was not detected in oral, conjunctival, preputial and rectal swabs from the two animals collected at several time points. Long-term (chronic) infection would be consistent with the relatively high number of viremic cats identified in epidemiological investigations, with the possible association of DCH with chronic hepatic pathologies and with what described with HBV in human patients.

Introduction

Hepadnaviruses have been identified in several animal species including primates, bats, rodents, birds and fish (Seeger, 2013; Wang et al., 2017). Chronic infections in humans of the prototype species, hepatitis B virus (HBV), increase the risk of liver diseases, including cirrhosis and hepatocellular carcinoma (HCC) (Karayiannis, 2017; Lok and McMahon, 2009; Seeger, 2013). In 2018, a novel

member of the family *Hepadnaviridae*, similar to HBV, was identified in a 7-year-old male neutered domestic shorthair cat, diagnosed with multicentric large B-cell lymphoma and with concomitant feline immunodeficiency virus (FIV) infection. On PCR screening with specific primers, the virus, named Domestic Cat Hepadnavirus (DCH), was identified in 10% of FIV-infected cats (Aghazadeh et al., 2018). The pathogenic potential of DCH is still uncertain, although hepadnaviruses are generally considered hepatotropic viruses (Karayiannis, 2017; Lok and McMahon, 2009; Seeger, 2013). In 2019, a multicentre study in USA, UK, Australia and New Zealand, explored the role of DCH in cats with chronic hepatitis and HCC (Pesavento et al., 2019). Liver biopsies from healthy cats and cats with hepatopathies were tested by PCR and in situ hybridization. DCH was detected in 43% (6/14) of chronic hepatitis cases and 28% (8/29) of HCCs, whereas cholangitis (n = 6), biliary carcinoma (n = 18) and normal liver (n = 15) all tested negative for DCH. Furthermore, in DCH-positive cases, the histologic features of inflammation and neoplasia, and the viral DNA localization, were strikingly similar to those seen in HBV-associated disease. These findings support an association between DCH infection and chronic hepatitis and HCC in cats (Pesavento et al., 2019).

We previously investigated the presence of DCH in cats, using an age-stratified population of animals (Lanave et al., 2019). On screening of 390 feline sera, DCH was detected in 10.8% of cats with a significantly higher prevalence (17.8%) in the sera of animals with a clinical suspicion of infectious disease, suggesting a possible association with FIV and feline leukaemia virus (FeLV) infections. For a subset (n = 20) of DCH positive samples, information on serum biochemistry parameters was available and 10/20 (50%) animals showed a profile consistent with liver damage. Also, in 7/10 animals with suspected hepatic disease, the virus load was $>10^4$ genome copies per mL, which is above the threshold for the risk of active hepatitis and liver damage from HBV infection in humans (Lanave et al., 2019).

The relatively high prevalence of DCH in feline sera, chiefly in 4-7 months old cats and in cats with retroviral infection, and the possible association of DCH with chronic liver pathologies, could be consistent with the aptitude of DCH to persist in some animals for a protracted period after acute

infection and eventually cause chronic infections, as observed in human patients infected with HBV (Fattovich, 2003; Seeger, 2013). In this study we performed longitudinal molecular screening of cats infected with DCH to understand if DCH may cause chronic infections in cats.

Material and methods

Clinical cases and sample collection

Surveillance in 390 household cats in Apulia region, Italy, was carried out in 2017-2019, on sera collected from two veterinary diagnostic laboratories. The sera had been submitted by veterinarian practitioners for routine testing during the course of veterinary diagnostic investigations. On molecular screening, 42 cats tested positive to DCH (10.8%) (Lanave et al., 2019). Longitudinal observation was possible for five DCH-positive cats, with the collaboration of veterinarian practitioners and consent from the owners, over 2-10 months after the initial diagnosis of DCH infection (Table 1). Blood samples were obtained by veterinarian practitioners in private clinics during follow-up examinations. The study was approved by the Ethics Committee of the Department of Veterinary Medicine, University of Bari (authorization 23/2018). All experiments were performed in accordance with relevant guidelines and regulations.

Cat 1. A 4-year old neutered male domestic shorthair (DSH) cat, with outdoor access, was presented in February 2017 for lethargy, dehydration and anorexia. The clinical signs had started 4 days before presentation and the cat recovered within 3 days. Abnormalities on serum biochemistry included elevations of creatine phosphokinase (CPK, 1704 International Unit [UI]/L; reference interval [RI] 10 – 140 IU/L) and alanine transaminase (ALT, 57 IU/L, RI 0 – 43 IU/L). On May 2018, the cat re-presented for bite-wounds and tested negative for FIV and FeLV on a point-of-care (POC) immunochromatographic test (Speed DUO FeLV/FIV, Virbac, Italy). Residual serum tested positive for DCH DNA by qPCR. Additional blood samples were collected 5 (October 2018) and 14 months (July 2019) after DCH infection was first detected.

Cat 2. A female 8-month-old DSH indoor-only cat presented in March 2019 for routine ovario-hysterectomy. Pre-operative POC testing for FIV and FeLV was negative and residual serum tested positive for DCH. A second blood sample was collected 4 months later (July 2019) at a follow-up examination.

Cat 3. A 7-month-old neutered male DSH cat, with outdoor access was presented in May 2018 for marked lethargy of 2 days duration. On a complete blood count there was a severe leukocytosis (20.7 x 10^3 /µL, RI 5.5 – 12 x 10^3 /µL) due to a mature neutrophilia (11,799 /µL, RI 3000 – 7000 /µL). The cat tested positive for both FeLV antigen and for FIV antibody and residual serum tested positive for DCH. Blood samples were collected at follow-up examinations in March and May 2019.

Cat 4. A 9-year-old neutered male DSH cat living outdoors presented in July 2017 for episodes of epistaxis. On rhinoscopy and histological examination chronic rhinitis was diagnosed and treated effectively with doxycycline. Abnormalities on serum biochemistry included elevations of ALT (233 IU/L; RI 0 – 43 IU/L) and the cat tested positive for FIV antibody but negative for FeLV antigen. DCH DNA was detected by qPCR on residual serum collected in February 2019. Blood samples were collected at follow-up examinations two (April 2019) and four months (June 2019) later. As the cat was living with cat #5, oral, conjunctival, preputial and rectal swabs were also collected in April and June 2019.

Cat 5. A 9-year old neutered DSH male outdoor cat, sibling to cat #4, had a history of an acute hepatopathy. It first presented in March 2016 with an acute onset of lethargy, anorexia and fever. Results of serology, haematology and serum biochemistry revealed moderate leukocytosis due to mature neutrophilia and the cat was FIV-seropositive and FeLV-seronegative. There were mild to moderate elevations of AST and ALT and bilirubin was markedly elevated (Table 2). During the subsequent four months liver enzymes were monitored frequently, revealing a persistent hepatopathy. The cat was jaundiced for several weeks around the time of first presentation, after which it was hospitalized and treated with intravenous fluid therapy and antimicrobials (ampicillin/sulbactam and cephazolin). Abdominal ultrasonographic examinations performed in April, June and August 2018,

showed structural hepatobiliary alterations including thickening of the gall-bladder wall. Fever was not noted in the animal after the 2016 acute episode. In February 2019 the serum was tested for DCH DNA for the first time and returned a positive result. Blood and swab samples, as described for cat #4, were collected in February, April, June and August 2019. Hepatic parameters on serum biochemistry remained abnormal throughout 2019 and in January 2020 (Table 2). The cat was found dead in February 2020 without signs of trauma and post-mortem examination was not permitted.

Molecular testing for DCH

Total DNA was extracted from the sera and swabs using QIAamp cadogen Pathogen Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. DCH was identified using quantitative PCR (qPCR). Briefly, 10 µL of the sample DNA or plasmid standard was added to 15 µL of master reaction mix containing 0.6 µmol/L of each primer (FHBV- for: CGTCATCATGGGTTTAGGAA; FHBV- rev: TCCATATAAGCAAACACCATACAAT) and 0.1 µmol/L of the probe (FHBV- prob: [FAM]TCCTCCTAACCATTGAAGCCAGACTACT [BHQ]). The reaction consisted of activation of the iTaq DNA polymerase at 95 °C for 3 minutes and 42 cycles of denaturation at 95 °C for 10 seconds and annealing-extension at 60 °C for 30 seconds (Lanave et al., 2019).

Evaluation of blood parameters

The sera of the animals testing positive to DCH during the follow-up period were submitted for hematochemical analyses. Complete blood cell count, including red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), white blood cells (WBC) and platelets (PLT), was performed on a blood sample collected into EDTA using an automated haematology analyser (HeCo Vet C, SEAC, Florence, Italy).

Serum proteins (eg. albumins, globulins), CPK, ALT, AST, ALP, gamma glutamyltransferase (GGT), bilirubin, and cholinesterase, were evaluated using commercial kits and an automatic UV spectrophotometer (SAT 450 AMS Alliance).

Screening for feline retroviruses

Screening for FeLV and FIV was carried out by practitioners with a POC immunochromatographic test (Virbac Test Speed DUO FeLV/FIV, Italy). Confirmatory testing for FeLV and FIV in our laboratories was performed using nested PCR and quantitative PCR on the blood, serum and oral swambs samples as described elsewhere (Morton et al., 2012; Quackenbush et al., 1996; Stiles et al., 1999).

Results

Three cats (animals #1, #3 and #4) tested negative using qPCR for DCH on the second and third screening tests performed a minimum of 2 months after DCH DNA was first detected (Table 1). As these animals tested negative on two consecutive occasions, they were not included in further investigations.

Two of the five cats (animals #2 and #5) tested positive to DCH in follow up monitoring (Table 1).

Animal #2 first tested positive on qPCR for DCH DNA on March 11 2019, with a viral load of $1.29x10^5$ DNA copies/mL. After four and a half months (July 25 2019), the cat tested positive for DCH with a virus titer of $1.48x10^4$ DNA copies/mL. No abnormalities were identified on cell blood count (CBC) or serum biochemistry. The animal was healthy, and resented handling so further serum samples were not obtained.

Animal #5 initially tested positive to DCH on February 4 2019, with a viral load of $4.48\ x10^5$ DNA copies/mL. Repeat testing returned positive results for DCH DNA on February 18 2019 ($2.09x10^6$ DNA copies/mL), on April 10 2019 ($9.68x10^5$ DNA copies/mL), June 12 2019 ($5.66x10^5$ DNA copies/mL), August 26 2019 ($1.64x10^5$ DNA copies/mL) and January 3 2020 ($1.32x10^6$ DNA copies/mL) (Table 1 and 2).

Table 2 shows the values of the biochemical main relevant parameters to the determination of liver injury of cat #5. Serum chemistry markers of hepatocellular injury (AST and ALT) or cholestasis (ALP) (Scott, 2008), were monitored (Figure 1). A persistent elevation in ALT values was identified

(Table 2) whilst elevations in ALP occurred twice, in June 2019 and August 2019 (Table 2 and Figure 1). Elevated AST values were recorded only from March to April 2016 (Table 2 and Figure 1). Furthermore, from May to June 2016 and from February to August 2019, high cholinesterase values were recorded (Table 2). Sera obtained prior to February 2019 were not available for virological investigations so the DCH-infection status of the cat at that time is unknown.

Oral, conjunctival, preputial and rectal swabs collected at four time points in February, April, June and August 2019 tested negative for DCH DNA. Since animal #4 was living with animal #5, we also screened swabs collected in April and June 2019 from animal #4, but DCH DNA was not detected in the samples. Also, the swabs of animal #4 and #5 tested negative for FIV on molecular analysis, even though both had previously tested seropositive for FIV.

Discussion

This is the first longitudinal study of DCH-infected cats. Out of five cats testing positive for DCH DNA during a sero-epidemiological investigation in Italy (Lanave et al., 2019) two cats (#2 and #5) remained positive for DCH DNA on retesting. The cats testing repeatedly negative were excluded from further observations, even if the absence of viremia must be interpreted with caution. Some HBV patients can enter into a status of chronic inactive carrier or, due to mutations in HBV genome, into a form of HBeAg-negative chronic hepatitis B, with viral DNA being present at low levels or not detectable at all in the blood (Coffin et al., 2019; Seeger, 2013). Multiple sampling points were available for one of these cats (#5), associated with monitoring following an acute hepatopathy in March-April 2016. Cat # 5, which was tested FIV positive in serological and molecular assay, tested also DCH positive on 5 repeat DCH tests over a total of 11 months. During this time, serum ALT remained persistently elevated consistent with ongoing hepatocellular injury. However, no definitive diagnosis for the hepatopathy was made and no conclusions can be drawn about the role, if any, for DCH in the hepatopathy. Cat # 5 was housed with a FIV-infected sibling, cat #4, that tested DCH-positive initially (February 2019), but was negative on repeat testing 2 and 4 months later.

Interestingly, cat #4 had also returned a previous elevated serum ALT (233 IU/L) in July 2017, during a clinical visit for episodes of epistaxis.

The route of DCH infection in the siblings is unknown. Transmission of HBV in humans occurs through blood and other body fluids and contagion can also occur during sexual contact and by maternal/fetal route (Seeger, 2013). Similar modalities of transmission might also be hypothesized for DCH. In cat #5 virus was detectable in serum throughout the 11 months observation period at titers ranging between $1.64x10^5$ and $2.09x10^6$ DNA copies/mL. In order to assess if whether cat #5 was shedding virus in body fluids/secretions, we collected oral, conjunctival, preputial and rectal swabs from the cat at 4 distinct time points (February, April, June and August 2019). However, DCH DNA was not detected in the swabs. Screening of other relevant biological samples (i.e. urine) was not possible. Also, swabs from animal #4 collected in April and June 2019 tested negative to DCH.

Body fluids such as saliva, semen, urine, sweat, and tears are potential sources of HBV transmission. Several studies have reported that HBV DNA in these body fluids can be detected by PCR (Heiberg et al., 2010; Hui et al., 2005; Kidd-Ljunggren et al., 2006; Komatsu et al., 2012; van der Eijk et al., 2004). Of these body fluids, however, only serum, saliva, and semen have been demonstrated to be infectious in humans or experimental animal models (Kidd-Ljunggren et al., 2006; Komatsu et al., 2012). In HBV patients with chronic infection, the titre of HBV DNA in urine, tears and saliva is correlated with the serum virus load, and when the HBV titer in serum is lower than 10^6 DNA copies/ml, the virus is barely, or not detectable in saliva and tears (Komatsu et al., 2012). If parallelism between HBV and DCH applies, cat #5 had serum virus titers below those expected for virus shedding in body fluids, which may account for the absence of detectable DCH DNA by PCR. These findings suggest that DCH viremic cats may not always shed the virus in body fluids. Future sampling cats with higher serum viral loads will be helpful for gaining a better understanding of DCH transmission routes.

Likewise, nucleic acids of FIV were detected neither in the swab samples nor in the sera of animal #4 and #5, both which were positive to FIV serologically. Cats infected with FIV often have low viral

loads and some laboratory cats with documented FIV infection have insufficient circulating provirus copies for molecular detection (Allison and Hoover, 2003; Lee et al., 1998; Sellon and Hartmann, 2012).

A challenge of HBV diagnostics in human patients is to define correctly the stage of HBV infection. For diagnosis of human viral hepatitis and for predicting the stage of infection, antigens, antibodies and viral genome are profiled/quantified and this information is coupled with haematological and blood chemistry tests (Seeger, 2013). In the case of DCH, only molecular diagnostics are currently available. In our study, qPCR was used to detect and quantify DCH in feline sera. The mean value of DCH viremia in sera of animal #5 was $9.26x10^5$ DNA copies per mL. The lower threshold for the risk of active hepatitis and liver damage in human patients infected with HBV is 10^4 viral genome copies per mL, equivalent to about 2000 IU (international unit)/mL (Seeger, 2013). Usually, high HBV DNA load in blood is found in acute infection, or in active chronic stages of disease (Seeger, 2013). A persistent ALT elevation had been documented in cat #5 over three years (Table 2). ALT is used as a key marker in the staging of HBV in human patients (Coffin et al., 2019; Seeger, 2013). Overall, we hypothesize that cat #5 was chronically infected with DCH. Acute hepatic pathology was documented in March-April 2016 with transient jaundice and increased hepatic markers (ALT and AST). Indeed, in cats, for ALT limited half-life in serum, a small increase is considered as an indicator of liver injury, while an increase in AST, which is more linked to hepatocytes-mitochondria, could represent a marker of the significant liver injury (Scott, 2008). However, differential diagnosis should include acute neutrophilic cholangitis and other hepatopathies, and it is uncertain whether the observed picture was primarily related to DCH. Moreover, the increased cholinesterase values could lead to a suspect of liver cirrhosis (Ramachandran et al., 2014) even if this suspicion has not been confirmed by further histopathological analysis. A limit of our observational study was that results of viral testing for cat 5# were not available prior to February 2019. Although the possible parallelism between HBV and DCH is intriguing, whether a correlation exists between DCH infection and liver damage needs to be firmly established. On screening of liver biopsies of DCH-positive cats by PCR

and in situ hybridization, DCH DNA was detected in 43% (6/14) of chronic hepatitis cases and 28% (8/29) of HCCs (Pesavento et al., 2019), implying long-term (chronic) persistence of DCH in liver tissues of cats.

In conclusion, we were able to identify prolonged infection by DCH in two out of five cats included in the observational study. We can hypothesize that a proportion of DCH-positive cats is likely to have long-term viremia. For one DCH-infected cat, which had a history of ongoing hepatocellular damage, it was possible to demonstrate the viraemia for 11 months, until death from unknown causes. These findings parallel between DCH infection in cats and HBV in humans. Importantly, DCH viraemia may pose unexpected risks in transfusion medicine. DCH, along with feline retroviruses, *Bartonella* spp. and feline hemoplasma, should be considered in the screening of donor subjects (Pennisi et al., 2015). Also, in order to assess the impact of DCH on feline health, it will be necessary to implement diagnosis of DCH when testing cats for other feline infectious agents.

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