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Case Report

Chronic equine hepacivirus infection in an adult gelding with severe hepatopathy

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Abstract

Background: Equine hepacivirus (EqHV) in equids represents the closest homologue to hepatitis C virus (HCV) infecting humans. A majority of HCV infected patients develop a chronic course of infection leading to liver fibrosis, cirrhosis and liver failure. However, in horses mostly transient mild subclinical infections are reported for EqHV to date. Objectives: EqHV can be involved in chronic liver diseases of horses. Methods: Biochemical parameters in serum samples were measured. Viral load was determined using qPCR. Next generation sequencing (NGS) of serum was performed. Liver tissue was stained with haematoxylin and eosin and analysed for viral RNA with fluorescent *in situ*-hybridization. Results: The horse showed symptoms of severe hepatopathy and was chronically infected with EqHV. Viral RNA was detectable in the liver during disease. To rule out other infectious agents NGS was performed and showed the highest abundance for EqHV. The identified virus sequence was similar to other circulating equine hepaciviruses. Conclusions: EqHV can be associated with liver disease in horses. Whether it causes the disease or contributes in a multifactorial manner needs further investigation.

Keywords: equine hepacivirus, hepatitis C virus, hepatopathy, liver, hepacivirus A.

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Introduction

EqHV (classified as hepacivirus A) in equids represents the closest homologue to hepatitis C virus (HCV) infecting humans (Pfaender *et al*. 2014). A majority of HCV infected patients develop a chronic course of infection leading to liver fibrosis, cirrhosis and liver failure (Hajarizadeh *et al*. 2013). However, in horses mostly transient mild subclinical infections are reported for EqHV (Ramsay *et al*. 2015). Furthermore, persistent infections and presence of viral RNA during hepatitis are described (Reuter *et al*. 2014; Scheel *et al*. 2015). Coinfection with EqHV and equine pegivirus was reported to have caused mild to moderate hepatitis in a horse (Pfaender *et al*. 2015). Additionally, an equine parvovirus was identified in association with liver disease in horses (Divers *et al*. 2018). In this study, we report a case of naturally occurring chronic infection with EqHV in a horse with signs of severe liver disease.

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Case description

In April 2017, a 6-year-old warmblood gelding was referred to the Clinic for Horses with a history of fever, anorexia and alterations of biochemical analytes of the liver. On initial presentation, the horse showed no clinical abnormalities, and the horse showed no signs of icterus or anorexia. Analysis of biochemical analytes identified elevated enzyme activities of glutamate dehydrogenase (GLDH), alkaline phosphatase (AP), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) and an increase in serum bile acid concentration as well as plasma fibrinogen concentration while total bilirubin was within normal limits (Table 1). No other horse was affected in the stable of origin. On transcutaneous abdominal ultrasonographic examination only dilated bile ducts and a heteroechogenicity of the hepatic parenchyma were noticeable. For histopathological examination, ultrasound guided percutaneous liver biopsy was performed. Histopathologically, intra- and interlobular hepatic fibrosis with bile duct hyperplasia and mild mixed inflammatory cell infiltrates was found (Fig. 1a). No organisms grew in bacterial culture of liver tissue. However, in the serum EqHV was detected by qRT-PCR with a titer of 1.1×10^5 RNA copies mL^{-1} serum (Table 1). qRT-PCR was performed as previously described (Burbelo *et al*. 2012; Reichert *et al*. 2017). Additionally, EqHV was detected in numerous hepatocytes using a fluorescence *in situ*-hybridization (FISH) technique (Fig. 1b). As a control liver tissue from an EqHV-negative horse was used (Fig. 1c). As the histopathological findings and the negative bacterial growth could not completely exclude concomitant bacterial hepatitis, antimicrobial therapy with trimethoprim-sulfadiazine (TMS) (30 mg kg^{-1} BWT [trimethoprim 120 mg g^{-1} and sulfadiazine 600 mg g^{-1}] q12 h PO) was initiated next to anti-inflammatory therapy with corticosteroids (prednisolone 1.5 mg kg^{-1} BWT q24h PO for the first 5 days, followed by prednisolone 1.0 mg kg^{-1} BWT q24h PO). The gelding was discharged from the hospital on day 5 after presentation.

Meanwhile, next generation sequencing (NGS) of serum was performed to identify the full length sequence of the genome for EqHV. Briefly, RNA was isolated, short read sequencing compatible RNA libraries were prepared and multiplexed sequencing was performed on a MiSeq instrument (4 234 567 per sample, 250PE). After depletion of host sequences, *de novo* assembly of contigs was performed (Fischer *et al*. 2014, 2015; Baechlein *et al*. 2015). Seven contigs were mapped to the reference strain NZP-1 (accession number JQ434001.1) and uploaded to GenBank (accession number MK775202). In order to rule out any strain specific characteristic for the disease development, the genome was compared to previously described sequences. The similarity plot shows closely related EqHV species (Fig. 2a).

Table 1. Biochemical parameters and EqHV quantification

	April 2017	May 2017	July 2017	January 2018	July 2018	Reference ranges
SBA (μmol L^{-1})	27.1	18.3	53.3	15.7	82.9	<12
ALP (U L^{-1})	752	615	823	277	1314	≤290
GGT (U L^{-1})	894	816	709	364	1427	≤20
GLDH (U L^{-1})	164.6	192.0	346.3	146.9	329.1	≤6
AST (U L^{-1})	289	303	283	266	384	<250
LDH (U L^{-1})	147	127	208	160	237	≤235
CK (U L^{-1})	133	179	157	106	174	<130
Total bilirubin (μmol L^{-1})	20.0	22.7	33.2	19	48.6	9.0–50.0
Fibrinogen (g L^{-1})	4.8	2.6	2.8	3.4	8.0	2.3–3.8
qRT-PCR	1.1×10^5	8.3×10^4	n.d.	2.4×10^4	5.2×10^5	

SBA, serum bile acids concentration; ALP, alkaline phosphatase; GGT, Gamma-glutamyltransferase; GLDH, glutamate dehydrogenase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; n.d., not determined.

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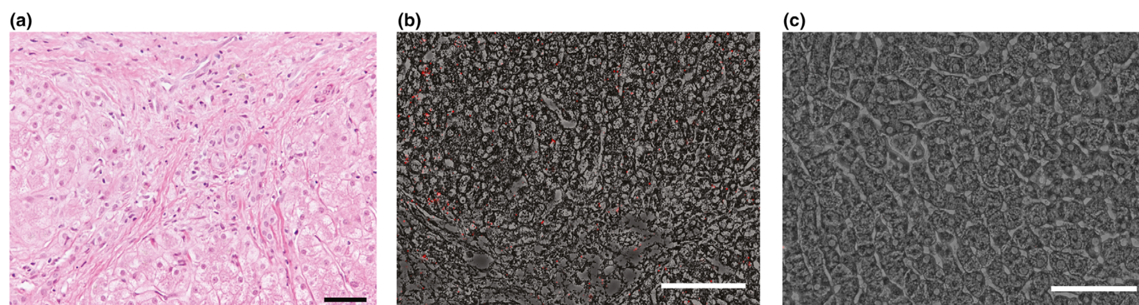

Fig. 1. Histopathology of liver biopsy (a) Histopathologically, an intra- and inter-lobular fibrosis was detected in hepatic biopsies. Furthermore a bile duct hyperplasia and mild inflammatory cell infiltrates within portal fields were evident. Hematoxylin and eosin (H&E) staining. Scale bar indicates 50 μm. (b) Intracytoplasmic equine hepacivirus (EqHV)-specific red positive signals were detected in numerous hepatocytes as determined by using fluorescent *in situ*-hybridization (Fast Red; ViewRNA TYPE 1 Probe Sets; ViewRNA™ ISH Tissue Assay Kit (1-plex) and ViewRNA Chromogenic Signal Amplification Kit; Thermo Fisher Scientific). Phase contrast and fluorescent microscopy. Scale bar indicates 100 μm. (c) No signal was observed when using the probe on liver tissue from an EqHV-uninfected horse. An identical method was used as for (b). Scale bar indicates 100 μm.

Furthermore, metagenomics of the tested sample revealed the highest relative abundance for EqHV (67.4% of all non-host reads) by far and the subsequent ranked pathogen (*Actinomyces ruminicola*, relative abundance 23.4% of all non-host reads) is not a known cause of disease in equids. Phylogenetic analysis revealed an overall close relation to other published full length sequences of EqHV worldwide (Fig. 2b). Analysis was conducted using the maximum-likelihood method by MEGA7 (Kumar *et al.* 2016) based on the General Time Reversible model (Nei & Kumar 2000).

Four weeks later, the horse was presented again to the equine hospital for follow-up examination. Repeated biochemistry revealed still increased liver enzyme activities and increased serum bile acid concentrations (Table 1). Ultrasonographic examination showed no improvement in hepatic abnormalities and the serum again tested positive for EqHV (8.3×10^4 RNA copies mL^{-1} serum). Medical treatment with TMS and corticosteroids was continued and it was recommended to present the horse for reexamination after 3 weeks.

In July 2017, the gelding was referred to the equine hospital because of a new episode of pyrexia, anorexia and lethargy. Meanwhile, antimicrobial and corticosteroid therapy were discontinued by the owner and the scheduled reexamination date missed. On presentation, the horse showed no fever or icterus but was lethargic, had decreased feed intake and showed weight loss. On ultrasonographic examination dilated bile ducts and heteroechogenicity of hepatic parenchyma were still present. Moreover, biochemical analytes were mainly unchanged (Table 1). Medical treatment with TMS and corticosteroids was initiated again. After discharge from the hospital the horse improved clinically according to the owner.

To rule out some toxic causes for liver damage, a mycotoxin analysis of the hay used for feeding was performed. Analyses revealed five measurable mycotoxins. Higher than expected concentrations of fusarenon X (1914 ppb), neosolaniol (380 ppb) and penicillic acid (357 ppb) were detected. The sample also contained lower levels of fusaric acid (44 ppb) and sterigmatocystin (10 ppb) (Table 2). None of these mycotoxins is a recognized cause of hepatotoxicity.

In January 2018, blood from the gelding was submitted by the owner. Enzyme activities of GLDH, GGT and AST as well as serum bile acids concentrations were improved (Table 1), but again the sample tested positive for EqHV with a decreased titre of 2.4×10^4 RNA copies mL^{-1} serum. The owner reported the horse to be afebrile and showing normal behaviour and appetite.

Six months later, the horse's condition deteriorated again and markedly elevated enzyme activities of GLDH, GGT and AST, serum bile acids

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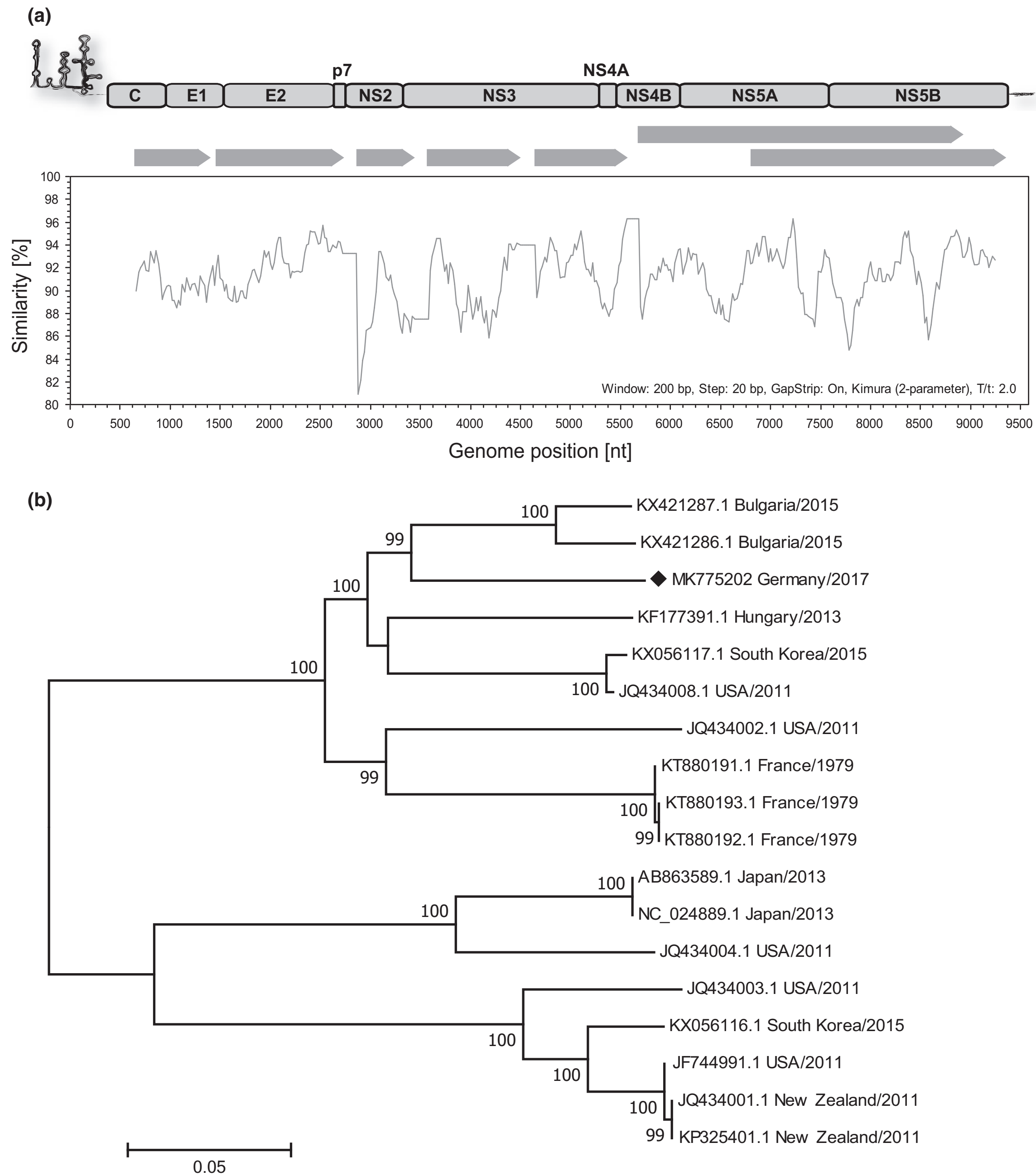

Fig. 2. Sequence analyses (a) Similarity plot of deep sequencing result compares the present EqHV-strain to available sequences for EqHV on nucleotide levels. The present EqHV-strain shows overall high similarities to other circulating strains. nt = nucleotide. (b) Maximum-likelihood phylogeny correlates the virus isolate sequence obtained using NGS with different published full length EqHV-sequences worldwide. Number of bootstrap replications was set to 1000. Bootstrap values < 70% are not shown. All positions containing gaps and missing data were eliminated. Black diamond indicates the sequence obtained in this study.

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Table 2. Mycotoxin analysis and concentration of the analysed hay sample

Mycotoxin groups	Amount, ppb
Aflatoxins (B1 + B2 + G1 + G2)	-
Ochratoxins (A+B)	-
Type B Trichothecenes	1914
Type A Trichothecenes	380
Fumonisins (B1 + B2 + B3)	-
Zearalenone Group	-
Fusaric Acid	44
Other Penicillium Mycotoxins	357
Other Aspergillus Mycotoxins	10
Ergot Toxins	-

Type B Trichothecenes = deoxynivalenol (DON) + 15-acetyl DON + 3-acetyl DON + fusarenon X + nivalenol + DON-3-glucoside; Type A Trichothecenes = T-2 + HT-2 + diacetoxyscirpenol (DAS) + neosolaniol; Penicillium mycotoxins = patulin + penicillic acid + roquefortine C + mycophenolic acid + wortmannin; Aspergillus mycotoxins = gliotoxin + sterigmatocystin + verruculogen.

concentrations (Table 1) and EqHV-RNA were detected. The serum sample tested negative for the newly identified equine parvovirus-hepatitis by qPCR. The horse was euthanized due to the poor prognosis and a *post mortem* examination was performed. A complete view of the whole liver (Fig. 3a) as well as the cutting side (Fig. 3b) showed highly altered liver tissue and micronodular liver cirrhosis was observed (Fig. 3c). Due to chronicity of lesions, the initial cause of this degenerative process could not be ascertained with the examination procedures conducted but infectious agents and non-infectious causes are both possible. Despite hepatic lesions, only mild inflammatory alterations were found histologically in the nasal passages, trachea, thoracic cavity, myocardium, lungs, kidneys and stomach, which were most likely of minor clinical relevance.

Discussion

In the presented case the horse showed clinical signs of hepatic disease at different time points. Despite treatment the liver disease progressed to the point that no further treatment was expected to improve the clinical state and euthanasia was recommended. Different causative agents were taken into consideration. Evidence was found for the

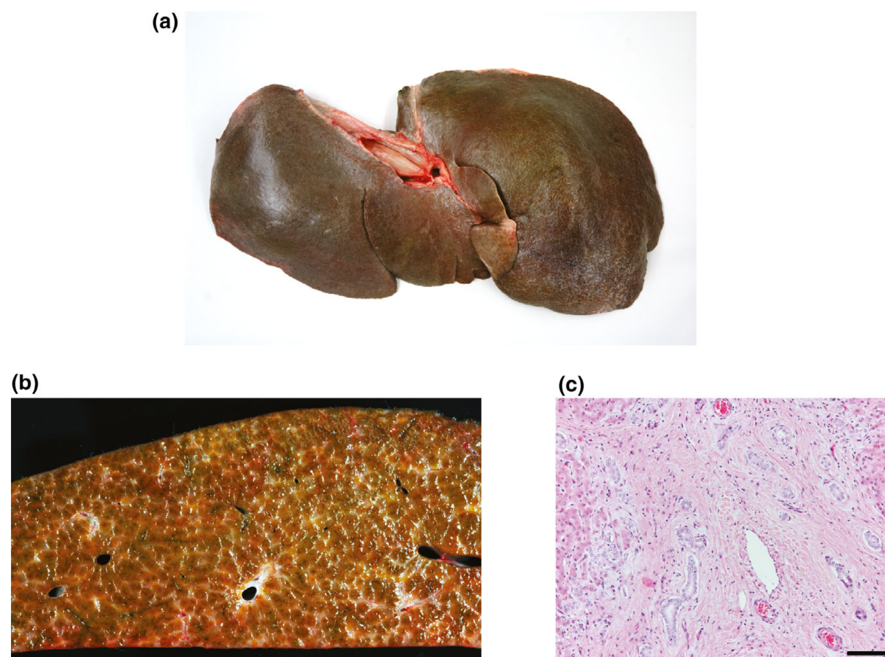

Fig. 3. *Post mortem* examination (a) (b) Macroscopic picture of liver, (a) whole liver, (b) cutting side (c) Histopathologically, a severe intra- and inter-lobular hepatic fibrosis was evident in liver samples obtained during necropsy. In addition, a bile duct hyperplasia and low numbers of inflammatory cells were present within portal fields. Haematoxylin and eosin (H&E) staining. Scale bar indicates 100 μm.

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presence of EqHV not only in serum by qPCR or NGS but also in biopsied liver cells by FISH. The close relationship of EqHV in horses to the HCV in humans raises the question of whether chronic infections in horses can lead comparably to liver cirrhosis and liver failure. Additionally, it has been reported that experimental infections have a mild effect on the liver (Pfaender *et al*. 2017). Although NGS revealed presence of other infectious agents like *Actinomyces ruminicola*, these are not known to cause any disease in equids. Moreover different mycotoxins were found, particularly fusarenon X, neosolaniol and penicillic acid. The contributing role of these toxins for the course of disease remains elusive although such hepatotoxicity is regarded unlikely in this case considering the severity of hepatopathy without an indication of involvement of other horses receiving the same feed sources. Experience of testing hay samples in the UK indicates that although many mycotoxins are commonly present in hay, fusarenon X is an unusual finding. Potentially, other toxic agents such as pyrrolizidine alkaloids in *Senecio jacobaea* could have induced liver damage but cannot be detected anymore. However, no indication of pyrrolizidine alkaloid ingestion was evident from the history and megalocytosis was not observed.

Altogether, EqHV might have contributed to a multifactorial disease development which caused severe liver disease in this horse.

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Ethics statement

The authors confirm that the ethics policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethics review committee approval has been received. The national guidelines for the Care and Use of Laboratory Animals were followed.

Contributions

B.T. and V.P. performed the lab work. J.E. performed the clinical work. N.F. performed the NGS from serum sample and the subsequent analysis. B.T, J.E, D.T, J.C. and E.S analysed the data. C.P, V.P. and W.B. performed the pathology including necropsy, histology and FISH. A.D. performed toxicologic analysis of the hay. B.T., J.E., E.S. and J.C. wrote the article. B.T., V.P., C.P., W.B., J.E., D.T. and E.S. prepared the figures and tables. All authors read and approved the final manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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