Research Article
Viral Hepatitis

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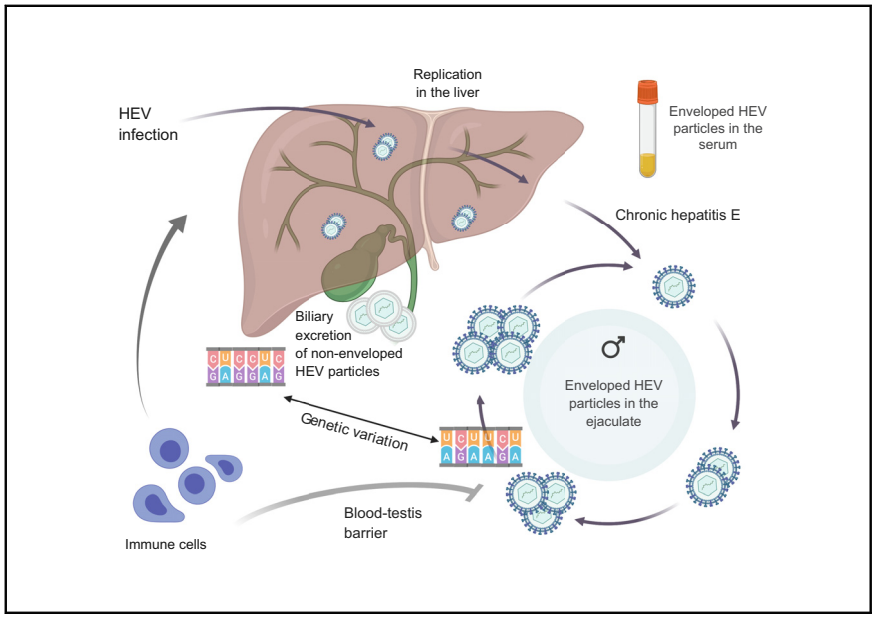
Hepatitis E virus persists in the ejaculate of chronically infected men

Graphical abstract



Highlights

- HEV genotype 3 particles were found in the ejaculate of patients with chronic HEV.
- Genetic variants differed between particles originating from the liver, compared to those originating from the male reproductive system.
- HEV shedding in the ejaculate continued for >9 months following the end of viremia.

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Lay summary

Enveloped hepatitis E virus particles could be identified by PCR and electron microscopy in the ejaculate of immunosuppressed chronically infected patients, but not in immunocompetent experimentally infected pigs or in patients with acute self-limiting hepatitis E.

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Hepatitis E virus persists in the ejaculate of chronically infected men

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Background & Aims: Hepatitis E virus (HEV) infections are prevalent worldwide. Various viruses have been detected in the ejaculate and can outlast the duration of viremia, indicating replication beyond the blood-testis barrier. HEV replication in diverse organs, however, is still widely misunderstood. We aimed to determine the occurrence, features and morphology of HEV in the ejaculate.

Methods: The presence of HEV in testis was assessed in 12 experimentally HEV-genotype 3-infected pigs. We further tested ejaculate, urine, stool and blood from 3 chronically HEV genotype 3-infected patients and 6 immunocompetent patients with acute HEV infection by HEV-PCR. Morphology and genomic characterization of HEV particles from various human compartments were determined by HEV-PCR, density gradient measurement, immune-electron microscopy and genomic sequencing.

Results: In 2 of the 3 chronically HEV-infected patients, we observed HEV-RNA (genotype 3c) in seminal plasma and semen with viral loads >2 logs higher than in the serum. Genomic sequencing showed significant differences between viral strains in the ejaculate compared to stool. Under ribavirin-treatment, HEV shedding in the ejaculate continued for >9 months following the end of viremia. Density gradient measurement and immune-electron microscopy characterized (enveloped) HEV particles in the ejaculate as intact.

Conclusions: The male reproductive system was shown to be a niche of HEV persistence in chronic HEV infection. Surprisingly, sequence analysis revealed distinct genetic HEV variants in the stool and serum, originating from the liver, compared to variants in the ejaculate originating from the male reproductive system. Enveloped HEV particles in the ejaculate did not morphologically differ from serum-derived HEV particles.

Lay summary: Enveloped hepatitis E virus particles could be identified by PCR and electron microscopy in the ejaculate of immunosuppressed chronically infected patients, but not in immunocompetent experimentally infected pigs or in patients with acute self-limiting hepatitis E.

Introduction

Hepatitis E, an infectious disease caused by the hepatitis E virus (HEV) occurs all over the world.[1] HEV is a positive strand RNA virus and frequently results in acute, often self-limiting, hepatitis.[1,2] The liver has been shown to be the predominantly affected organ in HEV infections, but several extrahepatic manifestations have been observed in this context.[3] Some of these seem to be caused directly by extrahepatic HEV replication in diverse organs.

Other extrahepatic manifestations seem to be caused by HEV-induced immune processes, presenting an overwhelming unspecific immune response affecting various tissues.[4,5] HEV replication outside of the liver and hepatocytes has been proven *in vitro* in experimentally infected neuronal and placental cell lines, as well as in cerebrospinal fluid, the kidney, placenta, intestine, lymph nodes and tonsils.[1,6–11]

In 2018, a Chinese group from the Kunming region presented a study indicating HEV genotype-4 replication in testis of experimentally infected macaques. Furthermore, this group described an unusually high detection rate of 28% for virus in ejaculate of infertile men that were infected with HEV genotype-4.[12] However,

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these findings could not be reproduced either in our center in a cohort of 87 infertile men,[13] or in a large Chinese study from Beijing investigating semen samples of 1,183 infertile men.[14] However, HEV RNA has been detected in ejaculate of Chinese pigs: an examination of 26 male pigs revealed an anti-HEV seroprevalence rate of 15.5%, while the proportion of detectable HEV in pig semen was far lower, at 4%.[15] Another animal study demonstrated the presence of HEV in testicular tissue in Mongolian gerbils experimentally infected with HEV genotype-4.[16] In contrast to these data, HEV has not yet been detected in semen of HEV genotype-3 (the predominant genotype in Western countries), infected men, or animals in areas where genotype-4 is endemic.

Other RNA viruses such as Zika, Ebola and SARS-CoV-2 virus have already been detected in the ejaculate of infected men,[17–19] suggesting that the blood-testis barrier allows for long-time viral testicular persistence in an immune privileged niche. While the blood-testis barrier can allow small particles such as viruses to easily transcend, it can prevent the much larger cells of the immune system from entering the testis.[20] Furthermore, it has been reported that the local immunosuppressive milieu and systemic immune tolerance are of central importance in preserving the immune privilege of the testis.[21]

Major aspects of testicular HEV replication, including the relevance in humans have not yet been studied. There is insufficient knowledge surrounding: (i) whether there is proof of HEV genotype 3 in the ejaculate and at which concentration; (ii) how long HEV can persist in the testis; (iii) whether HEV strains originating from the testis differ genetically or morphologically from those originating from the liver; and (iv) whether HEV may be a sexually transmitted disease. To address these points, we performed this prospective pilot study to examine the frequency kinetics and circumstances of HEV persistence in the ejaculate of chronically and acutely HEV genotype 3-infected men and of experimentally infected pigs. Furthermore, we assessed the morphological, genetic and virological characteristics of the HEV particles in the ejaculate in parallel with characterization of particles from serum and stool.

Materials and methods

In this prospective pilot study, we tested the ejaculate of 9 HEV-infected patients for presence of HEV RNA. While 3 of these patients (2 heart transplant recipients, 1 lymphoma patient) were immunocompromised individuals with chronic HEV infection persisting for more than 3 months, a further 6 immunocompetent individuals with acute self-limiting HEV infection were studied. All participating patients gave written informed consent. This study was approved by the local ethics committee (Reference Number PV5890) and conducted in accordance with the principles of the Declaration of Helsinki.

Virological analyses (qPCR)

Simultaneous PCR testing of blood, urine, stool and ejaculate was performed using a sensitive lab developed diagnostic quantitative PCR assay (qPCR; limit of detection 6 IU/ml, limit of quantification 24 IU/ml) on a fully automated real-time-PCR-system (Cobas6800, Roche). This PCR assay targeting the conserved open-reading frame (ORF)2-3 region of the virus has been normalized to the first WHO-standard.[22] The highly automated process on the Cobas6800 system reduces the risk of sample cross-contamination. HEV RNA positive full process controls (high and low) and the negative control were run with each PCR batch to ensure overall assay performance throughout the study. Duplex amplification of a full process control in each sample was performed to rule out PCR inhibition in every single sample.

Ejaculate samples of patients with chronic hepatitis E (Patient #1- 3) and acute self-limiting HEV infection (#5, #9) were tested for the presence of cytomegalovirus (CMV), Epstein-Barr virus (EBV) and herpes simplex virus (HSV) by PCR.

Testing for HEV in seminal plasma and semen

To determine whether HEV in the ejaculate originates in the prostate or in the testis, HEV-positive ejaculate samples were separated by centrifugation (10,000 U/min for 20 min) into seminal plasma (supernatant) and semen (sediment), and both were tested independently.

Genomic sequencing

To assess phylogenetic classification and compare the genomes of particles isolated from stool, liver and ejaculate, we amplified different regions, *i.e.* the hypervariable region (HVR) and RdRp (RNA dependent RNA polymerase) within ORF1 and the 5' end of ORF2/3 by PCR (primer information and cycling conditions are available upon request) followed by Sanger sequencing. Obtained sequences were checked for sufficient quality and nucleotide multiple sequence alignments (MSA) were constructed using the EMBL-EBI clustal omega online tool with pre-set parameters.[23] Phylogenetic reconstruction of MSA was performed using phylogeny.fr with the HKY85 substitution model and bootstrapping analysis.[24] For visual inspection and formatting we used MEGA X.[25] Detailed information are provided in the supplementary CTAT Table.

Viral particle density assessment

To assess and compare the density of viral particles in different bodily fluids (serum, stool and ejaculate), we performed isopycnic linear density gradient centrifugation, as described.[26] Serum and ejaculate samples and fecal suspensions were layered onto a linear 6–56.4% iodixanol (OptiPrep, Progen Biotechnik, Heidelberg, Germany) density gradient and centrifuged for 16 h at 41,000 rpm, 4°C in an SW60 Ti rotor (Beckmann Coulter, Krefeld, Germany). We collected 250 µl fractions from bottom to top and the density was determined by refractometry. Viral RNA was purified from the fractions using the QiAmp MinElute Virus Spin Kit (Qiagen). This was followed by quantitative PCR assay using the highly sensitive Cobas6800 (Roche) as described above.

Immune transmission electron microscopy

The samples were examined through transmission electron microscopy using a JEM 1400 microscope (Jeol, Tokyo, Japan) at 120-kV acceleration voltage. The samples were analysed either untreated or after treatment with sodium deoxycholate as detergent, as described in order to remove the lipid envelope from the particles.[27] HEV particles were identified by using the capsid protein-specific mouse monoclonal primary antibody G117-AA4 (elicited with an *E. coli*-expressed ORF2 of a HEV genotype 3 strain and kindly provided by Dr. J. Schenk, Hybrotec GmbH Potsdam, Germany) and detected by a secondary gold-labelled antibody. Negative staining of the labelled samples was done using uranyl acetate. As a control, labelling not with the primary antibody, but with secondary gold labelling was

performed in order to assess the degree of non-specific secondary antibody binding (not shown).

Infectivity of samples

To examine the infectivity of ejaculate, stool, urine and plasma specimens, HepG2/C3A cells were inoculated with the respective sample. HEV in the supernatant as well as in the cell lysate were quantified by qPCR as described elsewhere.[9] In brief, 0.1 ml of the stool sample were diluted in 5 ml MEM, vortexed and passed through a 0.45 μm filter. The other samples were diluted 1:10 in 5 ml MEM, vortexed and passed through a 0.45 μm filter. HepG2/C3A cells in a 96 well plate were then inoculated with 100 μl of the samples. After 6 hours, cells were washed and the media were replaced. The medium was then changed every 2 days and half of the supernatant was taken and stored at -80°C. After 10 days, the cells were washed with PBS and RNA was extracted from supernatant and lysed cells as previously described.[28] HEV particles derived from cell culture (Kernow p6_G1634R) were produced as previously described and served as positive control.[29] Extracted RNA was applied to SYBR Green based quantitative reverse-transcription PCR (qRT-PCR) using the GoTaq® 1-Step RT-qPCR System (Promega, Mannheim, Germany) with the following primer: 5'-GGTGGTTTCTGGGGTGAC-3' (sense) and 5'-AGGGGTTGGTTGGATGAA-3' (antisense).[30]

Experimentally infected pigs

Stored testicular tissue samples of experimentally HEV-infected pigs were tested for HEV by PCR. The experimental infections in pigs without analysis of their testes have previously been described.[31] The genotype used for the inoculum in the study was prepared from a highly HEV RNA positive liver of an experimentally infected wild boar. The original HEV genotype 3 strain used originated from a naturally infected wild boar hunted in Northern Germany.[31,32] The authority of the Federal State of Mecklenburg Western-Pomerania approved the animal experiments based on European Directive 2010/63/EU and associated national regulations. Domestic large white breed pigs were acquired from a commercial breeder (animal husbandry, Dummerstorf, Germany) and housed under containment conditions. Twelve male pigs were experimentally infected with HEV genotype 3 using inoculum at dilutions from 10^{-4} to 10^{-8}, corresponding to a dosage of up to 14,746 IU/dose; female pigs were excluded. A diagnostic qRT-PCR was performed targeting the ORF3 region according to an established protocol.[33] qRT-PCR was carried out with the QuantiTect Probe RT-PCR Kit (Qiagen, Germany) and the CFX96™Real-Time System (Bio-Rad Laboratories GmbH, Munich, Germany). In addition to this in-house PCR-assay, samples were also tested by the sensitive Cobas 6800 device in order to validate results.

Results

Experimentally infected pigs with acute HEV infection

Testes of HEV-infected pigs (n = 12) from a recent HEV titration study were analyzed for HEV-derived viral RNA.[31] HEV RNA was detected in serum samples of 8/12 pigs at more than 2 consecutive sampling time points. Viremia lasted up to 58 days post inoculation. HEV could not be found in testicular tissue in any of the 12 experimentally HEV-infected immunocompetent pigs, although 8/12 tested positive for HEV in their livers, 7/12 in various lymph node localisations, 6/12 in their gallbladders, 5/12 in their spleens and 8/12 in the faeces. For detailed data see Table 1.

Detection of HEV RNA in the ejaculate of patients with chronic hepatitis E

Two of three chronically HEV-infected patients tested repeatedly positive for HEV in their ejaculate (Fig. 1A,B). HEV RNA levels in the ejaculate were more than 2 logs higher than viral load in the serum. HEV concentrations in seminal plasma and semen did not differ for more than 1 log at 2 time points. Under treatment with ribavirin, HEV viremia disappeared in both patients, however, the ejaculate remained HEV RNA positive. In one of them, elevated HEV viral load in the ejaculate outlasted the duration of viremia for >9 months, as illustrated in Fig. 1A. Viral load in the ejaculate in the second patient strongly decreased but remained positive for more than 12 weeks longer than viremia. We then observed a dramatic increase of viral load in the serum, ejaculate, urine und stool, indicating a potential systemic reactivation of the HEV infection, as illustrated in Fig. 1B. In contrast, none of the 6 ejaculate samples from immunocompetent patients with acute self-limiting HEV infection (viremia range from 24 to 160,442 IU/ml) tested positive for HEV.

Cases of chronically infected individuals and acutely infected individuals

Clinical characteristics of patients studied are depicted in Table 2.

Patient #1 was a 48-year-old heart transplant recipient suffering from chronic hepatitis E (HEV genotype 3c). Elevated liver enzymes (2-fold above the upper limit of normal) were present for >24 months. By examining stored serum samples, we were able to prove that HEV viremia had persisted for more than 2 years. The immunosuppressive medication consisted of everolimus (through level 5–10 μ/L), enteric coated mycophenolate mofetil (360 mg twice daily) and prednisolone (5 mg daily). Reducing immunosuppression was not possible in this patient who had already suffered from repeated biopsy-proven heart transplant rejections. We initiated ribavirin therapy (600 mg/d, 7 mg/kg body weight) in 11/2018. HEV viral load in the blood decreased but was still slightly positive (<24 IU/ml) 6 weeks after ribavirin initiation. It became undetectable 7 weeks after initiation. HEV viral load in the ejaculate presented more than 2-log higher in comparison to blood and outlasted viremia for more than 9 months, as illustrated in Fig. 1A. The patient experienced a sudden episode of pain and hypoesthesia in his shoulders 12 weeks after initiation of ribavirin therapy (Fig. 1A). Magnetic resonance imaging and pathological electromyography revealed the diagnosis of neuralgic amyotrophy, a well-known extrahepatic manifestation associated with HEV infections. In 04/2019, HEV could be detected in his cerebrospinal fluid at a concentration of 24 IU/ml. At this time point, HEV-PCR tested negative in blood, whereas viral load was 275,000 IU/ml in the ejaculate and 18,700 IU/ml in the urine. After 7 months of prolonged ribavirin therapy, Patient #1 cleared the infection in all compartments. Viremia disappeared 2 months prior to the end of ribavirin therapy, whereas viral shedding in the ejaculate disappeared more than 5 months after cessation of treatment. See Table 2 and Fig. 1A for details. The neurological symptoms vanished within a period of 6 weeks.

Patient #2 was a 66-year-old male who developed chronic HEV (genotype 3c) infection in 2019 under the condition of a B-cell lymphoma (CD38+) with the type of chronic lymphatic

Table 1. Results of RT-qPCR analysis of selected tissue samples of experimentally HEV-infected pigs (groups 10^{-4} up to 10^{-8}).

	10^{-4}-group				10^{-5}-group		10^{-7}-group		10^{-8}-group			
	Pig1	Pig2	Pig3	Pig4	Pig5	Pig6	Pig7	Pig8	Pig9	Pig10	Pig11	Pig12
Faeces*												
Ct	28.4	34.3	34.2	29.4	26.9	23.9	27.1	32.3	no Ct	no Ct	no Ct	no Ct
copy	68.8	1.3	1.4	35.0	249.4	2,473.3	163.0	3.6				
Bile*												
Ct	24.4	31.5	30.1	24.1	20.8	20.2	24.6	25.1	no Ct	no Ct	no Ct	no Ct
copy	1,007.5	8.7	21.8	1,246.9	25,355.3	39,844.7	1,006.4	700.5				
Liver 1*												
Ct	24.5	29.5	25.7	22.0	29.4	23.8	no Ct	30.1	no Ct	no Ct	no Ct	no Ct
copy	1,884.9	38.6	727.6	13,142.2	41.9	3,226.2		7.8				
Liver 2*												
Ct	23.2	30.0	25.9	25.4	28.4	25.2	no Ct	32.2	no Ct	no Ct	no Ct	no Ct
copy	5,187.7	25.0	637.3	898.7	86.6	1,115.6		1.6				
Liver 3*												
Ct	24.3	29.6	29.4	31.6	29.1	25.2	25.9	no Ct	no Ct	no Ct	no Ct	no Ct
copy	2,202.3	14.7	40.8	7.3	50.6	1,120.3	219.8					
Liver 4*												
Ct	24.2	29.2	27.4	30.2	28.2	26.4	26.9	30.3	no Ct	no Ct	no Ct	no Ct
copy	2,354.4	46.2	191.2	21.5	103.4	423.1	99.3	7.0				
Gall bladder*												
Ct	30.0	no Ct	no Ct	32.1	28.1	25.9	24.9	29.1	no Ct	no Ct	no Ct	no Ct
copy	25.2			5.1	110.0	634.2	478.6	17.0				
Lnn. Hepat*												
Ct	31.9	no Ct	31.8	32.2	32.6	31.2	31.5	no Ct	no Ct	no Ct	no Ct	no Ct
copy	5.6		6.3	4.7	3.3	10.0	2.6					
Spleen*												
Ct	33.1	no Ct	32.8	32.2	33.4	31.9	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
copy	2.3		2.9	4.5	1.8	5.7						
Pancreas*												
Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
copy												
Ln mes*												
Ct	30.6	32.8	no Ct	32.1	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
copy	16.4	2.8		4.9								
Ln mand*												
Ct	no Ct	no Ct	33.4	no Ct	no Ct	31.4	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
copy			1.8			8.2						
Testis#												
Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct	no Ct
copy												

Viral copy numbers were calculated from CT values determined by qRT-PCR (HEV copies/µl RNA). No Ct ≥34.0 (=negative).
*Data from Dähnert *et al.* 2018.
#This study; HEV, hepatitis E virus; Ln, lymph nodes; qRT-PCR, quantitative reverse-transcription PCR.

leukemia which was initially diagnosed in 10/2011. The disease initially remained stable under therapy with fludarabine, cyclophosphamide and rituximab chemoimmunotherapy, followed by rituximab and bendamustin therapy due to nodal progress in 2014. After transformation to a diffuse large B-cell lymphoma in 2018, 3 cycles of R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone) were administered. From 12/2018 to 03/2019, HEV RNA tested positive repeatedly in blood, urine, stool and ejaculate, with a viral load of 1.6×10^6IU/ml in the serum and 1,300 IU/ml in the ejaculate. In 03/2019, we started the first ribavirin therapy (1,200 mg/d, 17 mg/kg body weight). The HEV viral load in the ejaculate exceeded the viral loads in blood, stool and urine easily, as illustrated in Fig. 1B. Under ribavirin therapy, HEV viral load in blood and stool decreased rapidly followed by a delayed decrease of virus concentration in the ejaculate and urine. After achieving HEV clearance in blood and stool 4 weeks after ribavirin initiation, urine remained positive at a low level. Unfortunately, we were forced to perform a stem cell transplantation due to progress of his lymphoma and interrupt ribavirin administration. After stem cell transplantation (07/2019) and discontinuation of ribavirin therapy, we observed a dramatic increase of HEV viral loads in ejaculate (1.4×10^6IU/ml) followed by an increase in blood (900,000 IU/ml) and urine (23,000 IU/ml), indicating a possible systemic reactivation. After starting a second course of ribavirin in 12/2019, we observed a clear decrease in HEV loads. The patient cleared viremia after 5 months of ribavirin re-treatment, and tested negative for HEV in the ejaculate between Months 8 to 11. See Table 2 and Fig. 1B for details.

Patient #3 was a 60-year-old heart transplant recipient, who suffered from chronic HEV (genotype 3i) infection for 7 years (see Table 2). Retrospectively, we were able to identify a HEV positive blood product in 2012 as the source of infection. After diagnosis of HEV infection in 2016, the patient received ribavirin (600 mg/d) for 6 months. At the time of diagnosis, the immunosuppressive regimen contained everolimus (through level 5-10 µ/L), mycophenolate mofetil (1,000 mg twice daily) and prednisolone (5 mg daily). The patient did not clear the infection under ribavirin, but showed a biochemical response as transaminase levels normalized. We therefore continued the ribavirin

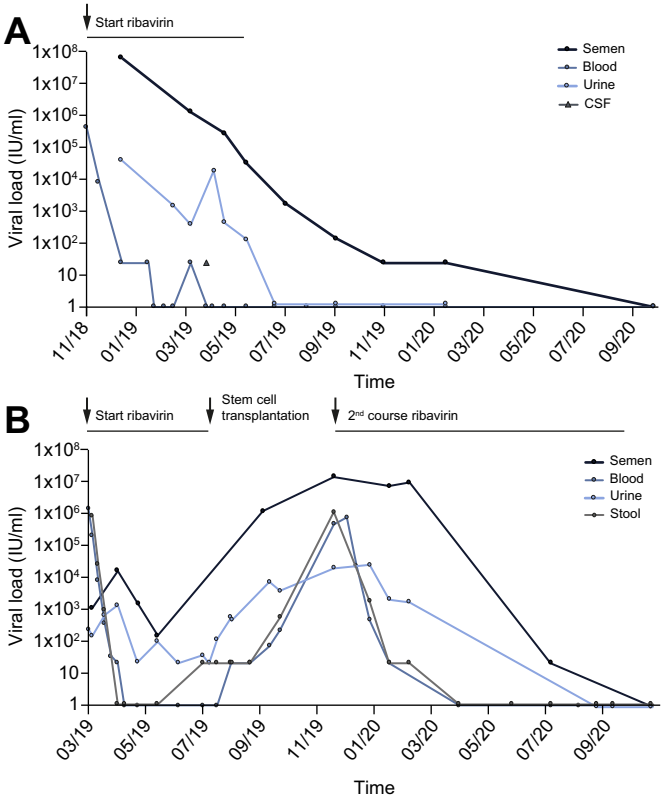

Fig. 1. Patients with chronic HEV infection. (A) Patient #1 is a 48-year-old heart transplant recipient with chronic HEV (genotype 3c) infection. (B) Patient #2 is a 66-year-old male lymphoma patient with chronic HEV (genotype 3c) infection. CSF, cerebrospinal fluid; HEV, hepatitis E virus.

treatment permanently at a low dose (200 mg/d). HEV infection relapsed, and we were not able to clear the infection. We could not detect HEV RNA in 2 ejaculate samples taken with a gap of 8 months, while HEV viral load in his serum was 16,000 IU/ml and 55,000 IU/ml at these time points.

Furthermore, we studied 6 immunocompetent patients (Patients #4-9, age ranging from 31 to 69 years old) with acute self-limiting HEV infection, as illustrated in Table 2. Four of these patients suffered from symptomatic acute hepatitis E (patient #6 resolving hepatitis E) and presented with marked elevation of alanine aminotransferase levels (up to 3,327 U/L, equal to >60-fold higher than the upper limit of normal), and a maximum viral load of 160,442 IU/ml. Two further patients had asymptomatic HEV infection (patients #4 and #5) which was detected in our blood donation unit (where routine screening for HEV is implemented). Both individuals had a low viral load of 24 IU/ml and presented without clinically apparent hepatitis. Genotyping could successfully be performed in reserve samples of 3 of these patients and demonstrated presence of HEV genotype 3c in Patients #7 and #9 and HEV genotype 3r (rabbit strain) in Patient #8.

Patients #1 and #2 tested positive for CMV in ejaculate samples, and negative for CMV in the serum at the same time point. Both patients tested negative for EBV and HSV in ejaculate samples. Ejaculate samples of Patients #3, #5 and #9 tested negative for CMV, EBV and HSV.

We tested the ejaculate for presence of HEV RNA of 2 further immunosuppressed patients who had recovered from chronic hepatitis E under ribavirin treatment more than 2 years ago. Notably, we did not detect HEV, CMV, EBV or HSV in the ejaculate samples of these patients.

Genomic sequencing of HEV variants

We amplified viral sequences from specimens of a chronically genotype 3-infected patient (Patient #2). Sanger sequencing of the ORF1 – HVR, the ORF1 – RdRp, the ORF2 and the ORF3 region of the HEV genome revealed 1 amino acid (aa) alteration between the dominant strain in the stool and the plasma, but 15 aa alterations of the dominant HEV-strain originating from the male reproductive tract (ejaculate sample) in comparison to the dominant strain deriving from the liver (plasma or stool sample). The phylogenetic trees show clustering of HEV genotype 3c in all sequenced regions (see Fig. 2A). An overview of aa alterations of the dominant HEV strain originating from the male reproductive tract (ejaculate sample) in comparison to the dominant strain deriving from the liver (plasma sample and stool sample) is provided in Fig. 2B. The MSA of aa sequences identified in the different compartments is shown in Table S1. Genotyping was further supported by the HEVnet Hepatitis E Virus Genotyping Tool v0.1 available at https://www.rivm.nl/en/hevnet.

Despite several attempts to perform sequencing analysis of Patient #1 at 2 different time points and different regions of the HEV genome including (i) ORF1 – HVR, (ii) ORF1 – RdRp, (iii) ORF2, and (iv) ORF3, it was technically not possible to obtain valid sequencing data.

Biophysical characteristics of HEV particles from various bodily fluids

We analyzed the density of HEV particles extracted from serum, ejaculate and feces of a chronically genotype 3-infected patient (Patient #2) by iodixanol density gradient centrifugation. HEV

Table 2. Clinical characteristics of studied patients at time point of ejaculate sampling.

Patient	Age (yr)	State of HEV infection	Immune status	ALT	ALT max.	HEV RNA (blood)	HEV RNA (ejaculate)
#1	48	Chronic	IS	29 U/L	628 U/L	<24 IU/ml	63,177.145 IU/ml
#2	66	Chronic	IS	82 U/L	109 U/L	240.038 IU/ml	1,275 IU/ml
#3	60	Chronic	IS	84 U/L	518 U/L	16.363 IU/ml	Neg
#4	46	Asymptomatic (blood donor)	IC	42 U/L	42 U/L	<24 IU/ml*	Neg*
#5	31	Asymptomatic (blood donor)	IC	72 U/L	74 U/L	<24 IU/ml*	Neg*
#6	69	Acute	IC	30 U/L	744 U/L	<24*	Neg*
#7	44	Acute	IC	1,891 U/L	1,891 U/L	13.378* IU/ml	Neg*
#8	54	Acute	IC	1,399 U/L	3,327 U/L	131.171 IU/ml	Neg
#9	43	Acute	IC	430 U/L	850 U/L	975 IU/ml	Neg

*In 4 of these patients the ejaculate sample was not available from the time point of blood testing, but within 1 week after. ALT, alanine aminotransferase; HEV, hepatitis E virus; IC, immunocompetent; IS, immunosuppressed.

RNA fractions of feces had a peak density of 1.26 g/ml, which was higher than that of HEV particles found in serum and ejaculate with a peak density of 1.11 to 1.12 g/ml (Fig. 3). Our data demonstrate that virions in the ejaculate are comparable to the enveloped virions in the serum but differ from structural features of non-enveloped particles in the stool.

Morphology of HEV particles from ejaculate and serum

Immunogold electron microscopy using an antibody directed against the HEV capsid protein identified HEV virions in the ejaculate and in the serum of Patient #2. In untreated samples, only very few particles showed gold labelling. In contrast, detergent-treated samples showed a high proportion of gold-labeled particles indicating that enveloped HEV particles were present in the samples. Regarding structural and morphological characteristics, virions in the ejaculate seemed to be intact and did not differ from viral particles in the serum (Fig. 4).

Assessment of infectivity in a cell culture system

In order to assess infectious properties of HEV RNA-positive ejaculate, human hepatoma cells were inoculated with specimens of all available bodily fluids. A molecular viral clone (Kernow-C1 p6) served as control in this HEV cell culture system as already described.[28] Notably, we could not observe infection of the cells with the HEV RNA-positive ejaculate, nor with serum, stool or urine of Patients #1 and #2 (data not shown).

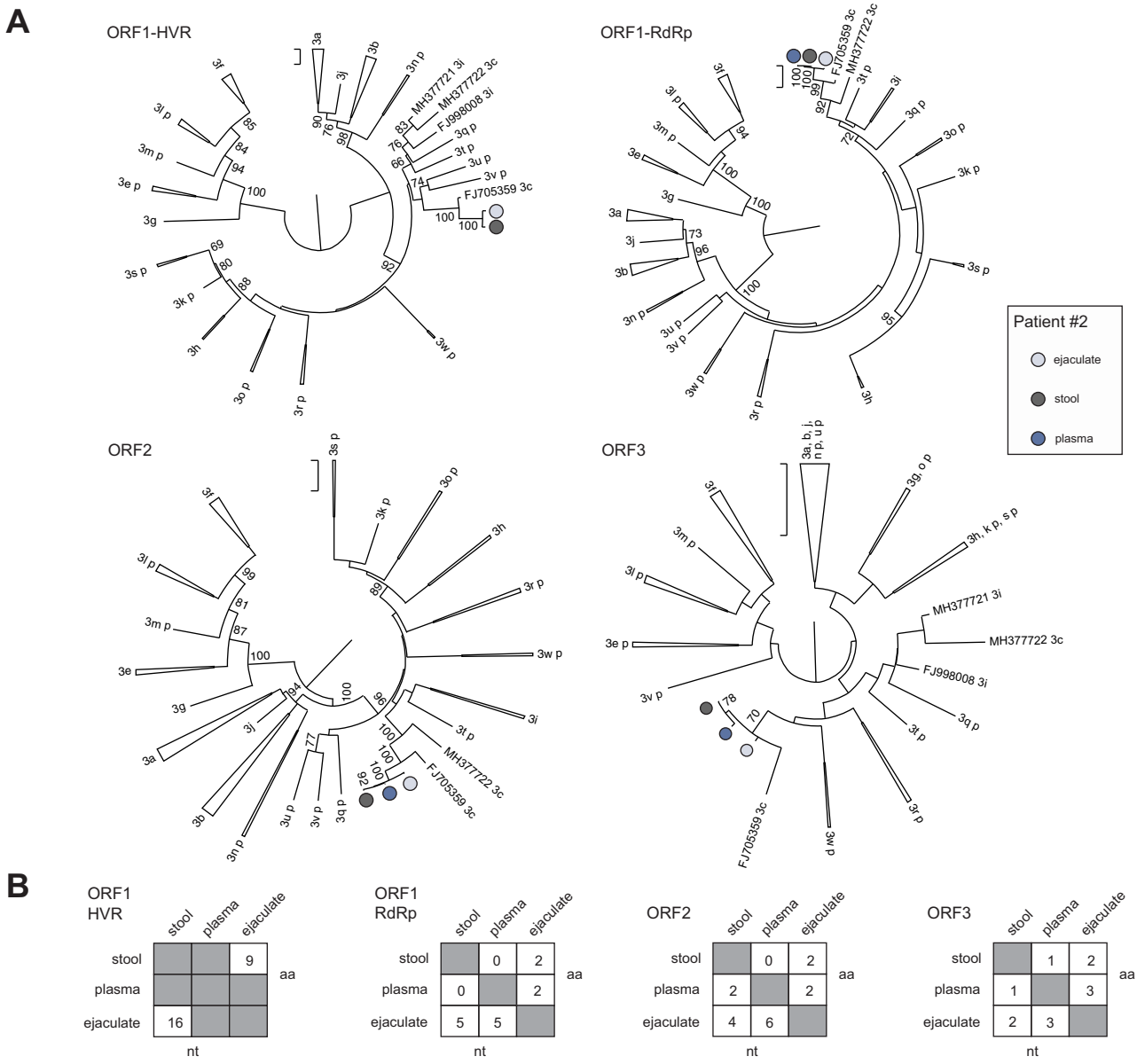

Fig. 2. Sanger sequencing of HEV genome. The ORF1 – HVR, ORF1 – RdRp, ORF2 and ORF3 of the HEV genome. (A) Phylogenetic trees show clustering of HEV genotype 3c in all sequenced regions. (B) Overview of amino acid alterations of the dominant HEV strain originating from the male reproductive tract (ejaculate sample) in comparison to the dominant strain deriving from the liver (plasma sample and stool sample) in Patient #2. The MSA of amino acid sequences identified in the different compartments is shown in Table S1. HEV, hepatitis E virus; HVR, hypervariable region; MSA, multiple sequence alignment; ORF, open-reading frame; RdRp, RNA dependent RNA polymerase.

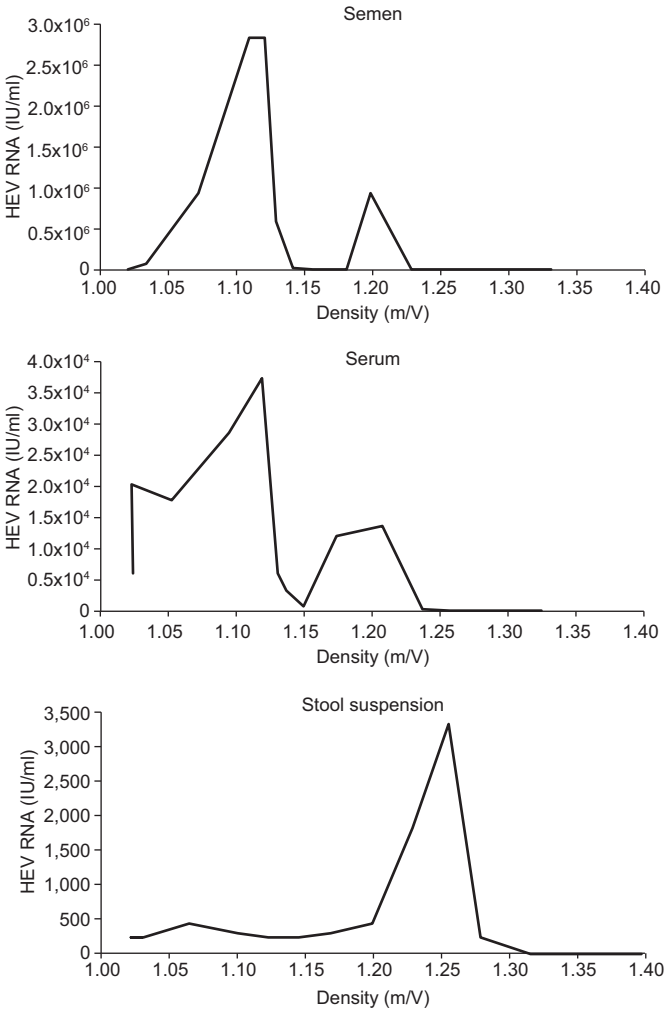

Fig. 3. Ultracentrifugation followed by PCR was used to determine the density of HEV particles from various compartments of Patient #2. HEV, hepatitis E virus; m/V, mass / Volume.

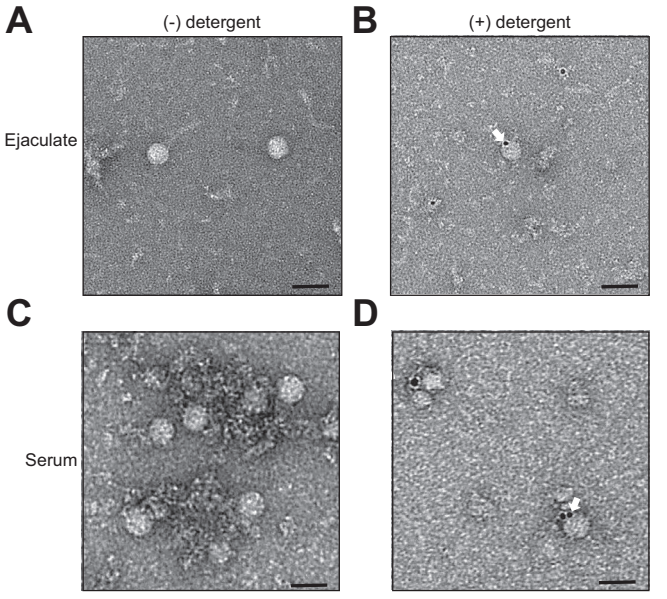

Fig. 4. Hepatitis E virions were identified by using a mouse monoclonal antibody specific for the capsid protein of HEV genotype 3 and a secondary gold-labeled antibody. (A) Virions in ejaculate without detergent treatment. (B) Virions in the ejaculate with detergent (sodium deoxycholate) treatment. (C) Virions in the serum without detergent treatment. (D) Virions in the serum with detergent (sodium deoxycholate) treatment; white arrows indicate the bound gold particles. HEV, hepatitis E virus.

Discussion

This is the first study demonstrating HEV genotype 3 viral particles in the ejaculate of chronically HEV-infected men. HEV genotype 3 virions could be identified by PCR and electron microscopy in human ejaculate. Interestingly, the presence of HEV in the ejaculate was limited to immunosuppressed chronically HEV-infected patients, but was not observed in immunocompetent experimentally infected pigs or in patients with acute self-limiting hepatitis E.

In 2 of 3 immunocompromised patients with chronic HEV genotype 3 infection, HEV could be detected in much higher concentrations in the ejaculate in comparison to the blood, demonstrating replication of HEV in the male reproductive system. HEV RNA was found in comparable concentrations in both fractions of the ejaculate: the seminal plasma and the semen. This indicates that these virions originate from the testis and the prostate. Alternatively, detection of HEV in the seminal plasma may be explained by free HEV RNA from broken HEV-infected sperm cells from the testis.

Notably, in Patient #2, detectable HEV RNA nearly vanished in his serum, stool, urine and ejaculate (Fig. 1B). After stem cell transplantation along with interruption of ribavirin treatment, this patient experienced a rise of HEV concentration in his ejaculate to 5-log higher than the low rate in his serum followed by reappearance of HEV in blood, urine and stool later. These findings led us to the assumption that HEV can replicate in the testis, resulting in spreading of particles into the body followed by systemic HEV reactivation in some cases. Thus, testis could be a niche representing a possible origin of HEV relapse in chronically HEV-infected patients, failing to achieve sustained virological response under ribavirin treatment, although it has previously been shown that ribavirin is able to pass the blood-testis barrier.[34] Whether these distinct replication sites are the source of individual viral populations, as suggested by aa differences in the majority of sequences identified by Sanger sequencing remains elusive. However, we cannot definitively rule out other niches of HEV replication, or the possibility of replication below the limit of detection.

Both patients tested HEV-RNA positive in the ejaculate and also tested positive for CMV in their ejaculate, while CMV in the serum was negative at the same time point. This further indicates that the testis may represent an immune privileged niche.

Nevertheless, our observation indicates for the first time, the value of ejaculate testing in HEV diagnosis and monitoring. This approach may allow early detection of chronically HEV-infected patients at risk of ribavirin treatment failure.

The spermatogenesis usually requires 64 days and sperm cells can survive for approximately 1 month in the male body. Thus, it seems plausible that pathogens replicating in the testis within 3 months after clearance of infection can be found. For example, Zika virus and Ebola virus were found in the ejaculate up to 62 days after hospital admission and up to 12 weeks post

infection.[35,36] However, in the case of immunosuppressed patients with chronic hepatitis E, we were able to find HEV in the ejaculate more than 9 months after clearance of HEV viremia.

In 6 immunocompetent individuals with acute HEV infection and 1 heart transplant recipient with chronic infection, we could not find any detectable HEV RNA in the ejaculate. This indicates that testicular HEV replication is not a common finding in acute HEV infections, but that it is limited to chronically infected men. Accordingly, HEV could not be detected in the testis of 12 experimentally HEV-infected immunocompetent pigs.

Both chronically HEV-infected patients with testicular HEV presence (Patients #1 and #2) suffered from genotype 3c, while the 1 heart transplant recipient with genotype 3i (Patient #3) twice tested negative for HEV in his ejaculate. This observation is only based on 3 patients, a cohort far too small to draw valid conclusions regarding various HEV strains and their ability to replicate in the testes. However, 2 other patients with acute hepatitis E were also genotyped as HEV 3c without the presence of HEV RNA in the ejaculate. Notably, 1 patient was identified as infected with HEV genotype 3r (rabbit strain). Genotype 3r is not common but has been described in European patients.[37]

Sanger sequencing showed that the dominant strain in the ejaculate differed from the strain in the stool on nucleotide and aa levels, and that it originated in the liver. The extent of the clinical relevance of these base pair exchanges in the HVR remains to be clarified. However, our observations are in line with those of Kamar *et al.* who assessed clonal HEV sequences in serum and cerebrospinal fluid and discovered a quasispecies compartmentalization in a chronically infected patient.[38] Drawing valid conclusions regarding the ability or inability of various HEV subtypes and variants to replicate in the male reproductive system will require a much higher number of patients, but certainly warrants further study.

Importantly, this study characterized biophysical properties and morphological features of HEV virions in the ejaculate in comparison to other bodily fluids. HEV is considered to be a quasi-enveloped virus, meaning that virions in the serum are enveloped by a lipid layer, whereas virions in the bile and stool are non-enveloped.[39,40] We observed that the density of HEV particles in ejaculate matches the density of particles in the serum, but differs from the density of HEV particles in the stool (Fig. 3). We could visualize HEV virions in the ejaculate using immune electron microscopy and we did not observe any structural or morphological deficiencies of the virions in the ejaculate compared to those in the serum.

The next step was to assess infectivity. Although virions in the ejaculate seemed to be intact, *in vitro* inoculation of hepatoma cells failed to score infectivity of HEV RNA positive ejaculate. However, HEV-positive serum, stool and urine of our 2 patients did not result in infection events in cell culture. Thus, we cannot draw valid conclusions regarding infectivity of HEV particles in ejaculate. The cell culture model, optimally adapted to a certain HEV variant is an ideal model to generate HEV particles and to evaluate the effects of possibly antiviral substances, but may have limitations when assessing the infectivity of random HEV strains.

Further animal experiments are needed to evaluate the infectivity of HEV particles. Based on these results, we can neither confirm nor definitely rule out evidence that HEV is a sexually transmitted disease. Therefore, until further data are available, the use of condoms in patients who are chronically infected with HEV should be discussed on an individual basis.

This pilot study had some limitations. First, the sample size is rather small. Furthermore, the study refers to a genotype 3 region and the relevant findings are limited to genotype 3c. Thus, valid conclusions referring to other HEV genotypes will require future studies. Furthermore, some of the immunocompetent individuals had low HEV viremia as a sign of recovery from acute HEV infection at the time point of inclusion in this study. They might have had a detectable HEV load in the ejaculate if they had been tested previously during the acute phase of hepatitis E. However, negative ejaculate testing in these patients confirms that HEV did not outlast viremia in ejaculate in high concentrations in contrast to the 2 immunosuppressed patients (#1 and #2).

The essential factors associated with the presence of HEV in the male reproductive system still need to be determined. Possible parameters allowing HEV to replicate in the testis could be the level of immunosuppression, the HEV viral load, the HEV genotype/subtype, or the duration of infection *per se*. Of note, both immunosuppressed patients with HEV in their semen suffered from genotype 3c, while 3e and 3f are the major genotypes in Germany.

This pilot study has led to several relevant implications for the field of Hepatology. Firstly, in line with Ebola, Zika virus and SARS-CoV-2, HEV can be detectable in the ejaculate in more than 2 orders of magnitude higher concentrations than in the serum in some patients with chronic infection, but not in those with acute infection. It seems that the virus avoids being eliminated by the human immune response by utilizing the immunological niche of the male reproductive system. Secondly, HEV can persist in the ejaculate for more than 9 months after viremia. Thirdly, dominant viral strains originating from the testis are different than those originating from the liver/bile, but density gradient and electron microscopy showed that seminal HEV particles are intact and seem to be enveloped. Finally, our cell culture model failed to prove infectivity of HEV RNA-positive ejaculate. It also failed to prove infectivity of HEV particles originating from other compartments.

Future studies are clearly needed to further elucidate and fully understand the impact of HEV replication in the male reproductive system. In addition, further study of the infectivity of HEV virions derived from different bodily fluids is required.

Abbreviations

CMV, cytomegalovirus; HEV, hepatitis E virus; HSV, herpes simplex virus; HVR, hypervariable region; MSA, multiple sequence alignment; ORF, open-reading frame; qRT-PCR, quantitative reverse transcription PCR; RdRp, RNA dependent RNA polymerase.

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Conflict of interest

Authors declare that they have no conflict of interests regarding this manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contribution

TH and SP developed the concept, coordinated the study and wrote the manuscript. RL, KH, JS, WV, FA, MR and ML assisted with collection of clinical data and testing of samples. JW, MG, ME, DT, LD, AS, ML, RJ, AG, RR, EH and ES performed laboratory experiments and procedures. TH and SP supervised the data collection and reviewed the drafts. JS, AWL, MMA, EH, ML and ES revised the manuscript for important intellectual content. All authors reviewed the final manuscript.

Data availability statement

The datasets generated and analyzed during this study are available from the corresponding author on reasonable request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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