# Extra-hepatic replication and infection of hepatitis E virus in neuronal-derived cells

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**Abstract**

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans and a member of the genus *Orthohepevirus* in the family *Hepeviridae*. Infection usually leads to acute hepatitis which can become fulminant, particularly among pregnant woman and in patients with preexisting liver disease, or may evolve to a chronic state, especially in immunosuppressed individuals. HEV has been shown to produce a range of extra-hepatic manifestations including aplastic anaemia, acute thyroiditis, glomerulonephritis as well as neurological disorders such as Guillain-Barré syndrome, neuralgic amyotrophy and encephalitis. The pathogenesis of these neurological injuries remains largely unknown and it is also uncertain whether or not HEV can directly infect neuronal cells. In this study, we investigated whether HEV is capable of completing the viral life cycle in human neuronal-derived cell lines such as neuroepithelioma (SK-N-MC), desmoplastic cerebellar medulloblastoma (DAOY), glioblastoma multiforme (DBTRG), glioblastoma astrocytoma (U-373 MG) and oligodendrocytic (M03.13) cells. Following transfection of these cells with HEV *Gaussia* luciferase reporter virus, all tested cell lines supported HEV RNA replication. Furthermore, extra- and intracellular viral capsid was detected by an HEV antigen ELISA as a marker for virus assembly and release. Permissiveness for HEV cell entry could be demonstrated for the oligodendrocytic cell line M03.13. In conclusion, these results indicate that HEV tropism is not restricted to the liver and HEV can potentially complete the full viral life cycle in neuronal-derived tissues explaining neurologic disorders during HEV infection.

## Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans and was recently classified as a member of the genus *Orthohepevirus* within the *Hepeviridae* family (1). HEV is a small, non-enveloped virus which carries a positive-sense, single-stranded, 7.2 kb-long capped RNA genome encoding three open reading frames (ORFs): ORF1 encodes a non-structural polyprotein required for viral replication, ORF2 is translated into the capsid protein which is responsible for virus assembly and binding to host cells and ORF3 encodes a protein with multiple functions including virus egress (2). Four genotypes of HEV have been identified to infect humans. Fecal–orally transmitted HEV genotypes 1 and 2 are a major cause of acute hepatitis often caused by contaminated drinking water in developing countries. Genotypes 3 and 4 are mainly zoonotic infections transmitted via contaminated food (3). In many animal species including swine, deer and wild boar different HEV isolates have been identified (4, 5). Hepatitis E is in most cases an asymptomatic, self-limiting disease and acute infections usually require no treatment. Symptoms of acute hepatitis include abdominal pain, jaundice, fever, fatigue and liver enzyme elevation (5). However, patients with underlying liver diseases and pregnant women might develop fulminant hepatitis leading to liver failure and death. In pregnant women mortality rates as high as 25% have been described (6). Chronic infections have so far mainly been observed for genotype 3 infections in immunosuppressed individuals including solid organ transplant recipients and human immunodeficiency virus (HIV)-infected patients. The first strategy to control HEV in chronically infected transplant recipients is the reduction of the immunosuppressive therapy (7). Also ribavirin (RBV) monotherapy has been successfully used for the treatment of chronic (8, 9), but also in severe acute HEV infections (10, 11). One prophylactic vaccine has been registered in China in 2011 (Hecolin®) (12), but is not available in other regions.

In addition to the characteristic hepatic affection, many extra-hepatic manifestations have been described in association with acute and chronic hepatitis E, including neurological

injuries, pancreatitis, acute thrombocytopenia, aplastic anemia and renal diseases (13). Most importantly, neurological disorders such as Guillain-Barré syndrome (GBS), brachial neuritis, encephalitis, ataxia/proximal neuropathy and neuralgic amyotrophy (NA) have been documented in about 5% of HEV infections (14-16). Recent studies from two European countries, the Netherlands and the United Kingdom, documented that 5% of patients with GBS and 10% of patients with NA had an associated acute HEV infection (17, 18). So far, these neuronal injuries have mainly been described for infections with genotype 3. However, one report about a patient from Bangladesh also pointed to genotype 1-associated GBS (19) and recently a patient from Japan was reported for the first time with genotype 4 HEV-associated neuropathy (20). HEV RNA has been found in the cerebrospinal fluid (CSF) of some but not all patients experiencing neurological manifestations (15, 21, 22) and a comparison of clonal sequences in the CSF and serum showed quasispecies compartmentalization implicating the existence of HEV in neuronal cells (22). However, the pathogenesis of these extra-hepatic manifestations remains largely unknown and neuronal tissues have so far not been described as an extra-hepatic site for HEV RNA replication and infection.

In this study, we investigated a set of human neuronal-derived cell lines for their capability to facilitate the propagation of HEV. First, we tested whether neuronal cell lines allow intracellular replication of genotype 1 and 3 HEV RNA following transfection with subgenomic reporter replicons or with full-length HEV. Further, virus assembly and release was investigated by the detection of the capsid (ORF2) in the supernatant of transfected cells and entry after infection of neuronal cells with cell culture-derived virus stocks. Our results demonstrate that HEV tropism is not restricted to the human liver as HEV could complete the full viral life cycle in M03.13 oligodendrocytes, which supports the observations of HEV-associated neuropathologies.

## Material and Methods

*Plasmids:*

The plasmid constructs of full-length HEV genotype 3 Kernow-C1 strain (clone p6; GenBank accession number JQ679013) and the two subgenomic *Gaussia* luciferase-encoding plasmid constructs of genotype 1 Sar55/S17 strain (based on clone pSK-E2, GenBank accession no. AF444002, with the insertion of S17 sequence in the hypervariable region) and Kernow-C1 p6 were described previously and were used for *in vitro* transcription (23-25). Capping was performed using Ribom7G Cap Analog (Promega, Madison, WI, USA).

*Drugs:*

Ribavirin was purchased from either Sigma Aldrich (St. Luis, MO, USA) or ICN Pharmaceuticals (Costa Mesa, CA). A stock solution was prepared in dimethyl sulfoxide (DMSO) and stored at 4°C. Concentrations of 50 μM or 100 μM were used in line with previous studies (26).

*Cell Culture:*

SK-N-MC cells were obtained from Rainer Niedenthal, Institute of Biochemistry/Physiological Chemistry, Hannover Medical School. U-373 MG, DAOY and DBTRG cells were obtained from Arend Koch, Charité Berlin, Germany. The oligodendrocytic (glial) hybrid cell line M03.13 was purchased from Cedarlane Laboratories Ltd. (Burlington, Ontario, Canada). HepG2/C3A were a kind gift from Luc Verschaeve (Scientific Institute of Public Health, Brussels, Belgium). These human liver cell lines and HepG2 cells (ATCC HB-8065) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS; Gibco).

HepG2 cells were further grown on rat collagen-coated (SERVA Electrophoresis GmbH, Heidelberg, Germany) petri dishes. DAOY and M03.13 cells were cultured in Dulbecco`s Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% FBS Gold (GE Healthcare Europe GmbH, Freiburg, Germany), 1% non-essential amino acids (Life Technologies), supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin (Life Technologies) and 100 IU/ml penicillin (Life Technologies) (DMEM complete). For culturing the DBTRG and U-373 MG cell line, DMEM complete was additionally supplemented with 1 mM of sodium pyruvate (Life Technologies). SK-N-MC were cultured in Advanced Minimum Essential medium (MEM, Life Technologies) and supplemented with 10% FBS Gold, 1% non-essential amino acids, supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin. Cells were kept at 37°C in a humidified 5% $CO_2$-incubator.

*HEV replication assay*

Cells were transfected via electroporation in accordance to previous reports (27). In brief, 6-9 x $10^6$ cells were resuspended in 400 µL Cytomix containing 2 mM ATP and 5 mM glutathione and mixed with 3-7 µg of the respective *in vitro* transcribed HEV RNA. After electroporation with a Gene Pulser system (Bio-Rad, Munich, Germany), cells were immediately transferred into 10-15 ml of the respective culture medium. Cell suspension was seeded into 12-well plates (1 ml per well, electroporation with subgenomic HEV RNA) or 24-well plates (500 µL per well, electroporation with full-length HEV RNA), partly provided with glass coverslips. Four hours post transfection (h p. t.), medium was replaced and fresh culture fluid or medium supplemented with 50 or 100 µM of RBV was added to the cells. At 4, 24, 48, 72 (and 96) h p. t., the supernatant, lysed or fixed cells or cells subjected to freeze and thaw cycles were further analyzed.

*HEV Gaussia Luciferase Assay*

At 4, 24, 48 and 72 h post transfection *Gaussia* luciferase activity was measured in supernatants of transfected cells. Therefore, 20 µl of supernatant per well were transferred to a 96-well white, flat-bottom microplate. Luminescence was determined using a microplate luminometer (CentroXS$^3$ LB 960, Berthold, Freiburg, Germany) after dispensing of 60 µl Coelenterazine at a dilution of 1:1000 in phosphate buffered saline (PBS), followed by shaking for 1 s and reading for 10 s.

*Indirect Immunofluorescence*

Five hundred µl of transfected cells suspension was seeded on glass coverslips in 24-well plates. After 48 h, cells were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS. After blocking of non-specific binding with 5% goat serum (Sigma Aldrich) in PBS, immunostaining of ORF2-encoded capsid protein was performed. Therefore, a mouse antibody directed against ORF2-encoded protein (aa 434-457, clone 1E6, LifeSpan BioSciences, Inc., Seattle WA) was added at a dilution of 1:1,000 in PBS supplemented with 5% goat serum. A fluorescently-labelled goat antibody (AlexaFluor 488, Life Technologies) was used at a dilution of 1:1,000 in PBS with 5% goat serum to detect bound primary antibodies. DNA was labelled with DAPI (4′,6′-diamidino-2-phenylindole) (Life Technologies).

*Detection of HEV antigen*

At indicated time points post transfection, the cell culture supernatant and cells were harvested. The cells were scraped in 1 ml of fresh culture fluid and lysed by three freeze and thaw cycles. Subsequently, the antigen content in cell lysates and supernatants was determined by a commercially available enzyme-linked immunosorbent assay (ELISA) (Wantai Biopharmaceutical Inc., Beijing, China). In this assay, antigen (ORF2-encoded capsid protein) is captured using goat polyclonal anti-HEV antibodies and antigen detection is

visualized with enzyme-linked monoclonal antibodies against ORF2-encoded capsid protein. One hundred µl of (diluted) sample (1:1-1:30 in PBS) was added for each reaction and the test was performed in accordance to the manufacturer's instructions.

*Quantitative detection of HEV RNA*

Cellular RNA was isolated from transfected, lysed cells at indicated time points post transfection using a Nucleo Spin RNA Kit (Macherey-Nagel, Düren, Germany) according to the manusfactorer's instructions. Concentrations of the isolated RNA were quantified spectrophotometrically. Subsequently, cDNA was generated from extracted RNA using a PrimeScript First Strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan) according to the protocol provided by the manufacturer. Quantitative PCR was performed with the LightCycler 480 system (Roche, Basel, Switzerland). The primer sequences for amplification of HEV RNA target the ORF1 region and have been described previously (28). A standard curve was created for each run using a HEV genotype 3 Kernow-C1 p6 plasmid DNA template to calculate the total amount of HEV RNA per well of transfected cells.

*Infectious virus yield assay*

Infectious virus was obtained from plasmid encoding full-length genotype 3 Kernow-C1 p6 HEV as described before (26). Cells were seeded in 6-well plates at 5 x $10^5$ cells per well in 2 ml of DMEM and were incubated at 37°C. After 24 h, culture medium was removed and cell layers were inoculated with infectious p6 virus at 3 x $10^7$ RNA copies/ml in 1 ml of medium with or without RBV (final concentration: 50 µM) and then incubated at 35°C. After 5 h, the inoculum was removed, cell layers were washed 3 times with 2 ml of PBS, and 2.5 ml of medium with or without RBV (50 µM) was added to each well. After 1 h, a 150-µl sample was taken and stored at -80°C. One ml of the medium was removed every 2-3 days, stored at -80°C until RNA extraction and 1 mL of fresh medium (with or without RBV) was added to

each well. Viral RNA was extracted with the NucleoSpin RNA virus kit and determined by quantitative real-time polymerase chain reaction (qRT-PCR). Quantification of total HEV RNA was performed essentially as described with forward primer 5'-GGTGGTTTCTGGGGTGAC-3', reverse primer 5'-AGGGGTTGGTTGGATGAA-3' and probe 5'-FAM-TGATTCTCAGCCCTTCGC-MGBNFQ-3', labeled with 6-carboxyfluorescein (FAM) at the 5' end and with a minor groove binder (MGB) at the 3' end (26).

## Results

### Neuronal-derived cell lines support HEV subgenomic RNA replication

First, we examined whether human neuronal cell lines support extra-hepatic subgenomic RNA replication of HEV. To this end, glioblastoma multiforme (DBRTG), neuroepithelioma (SK-N-MC), desmoplastic cerebellar medulloblastoma (DAOY), glioblastoma astrocytoma (U-373 MG) and oligodendrocytic (M03.13) cell lines were transfected with a genotype 3 replicon based on the strain Kernow-C1 p6 (suppl. Table 1). In this construct part of the ORF2 is replaced by a *Gaussia* luciferase which is secreted following productive RNA replication (24). The human hepatocellular carcinoma cell line HepG2 served as a positive control and RBV was used as a potent HEV RNA replication inhibitor. As depicted in Figure 1A, HepG2 cells showed efficient accumulation of reporter activity as marker for viral replication over time which could be blocked by RBV (Fig. 1A). All neuronal-derived cell lines evaluated supported HEV replication with an increase of *Gaussia* luciferase activity over the inhibitor control and time, but differences in the efficiency of RNA replication were observed (Fig. 1). The oligodendrocytic cell line M03.13 allowed HEV multiplication as high as in the HepG2 cells (Fig. 1F), while the other cell lines (DBRTG, SK-N-MC, DAOY and U-373 MG) showed lower replication competence with about 0.5-1.5 $\log_{10}$ reporter activity over the RBV control at 72h (Fig. 1B, C, D and E). We next tested HEV replication efficiency of a genotype 1 (strain Sar55 pSK-E2/S17) replicon in the different neuronal cells (Fig. 2), since one previous study described a neurological manifestation of HEV genotype 1 in a HEV patient (19). For HepG2 and M03.13, HEV replication levels were comparable to those of genotype 3 subgenomic HEV results (Fig. 2A and F). However, all other cell lines displayed none to very low genotype 1 replication capacity (<0.5 $\log_{10}$ reporter activity over RBV control after 72h) demonstrating a genotype-dependency for some of the neuronal cell lines (Fig. 2). In summary, HEV subgenomic genotype 3 and to some extent also genotype 1 replicons were able to replicate in different neuronal-derived cell lines.

**Replication of full-length HEV RNA in neuronal-derived cell lines**

Next, we evaluated whether the different neuronal cell lines would also support replication of full-length HEV RNA by transfection of the genotype 3 HEV p6 clone not harboring a reporter gene. To visualize HEV antigen expression, we performed immunofluorescence staining of the structural capsid protein (ORF2-encoded) 48h post transfection (Fig. 3A). For the human liver cell line HepG2 and all neuronal-derived cell lines ORF2-positive cells were detectable, which was not the case for RBV treated cells. For quantification of the HEV replication efficiency in each cell line a qRT-PCR was performed (Fig 3B-G). As shown in Fig. 3G and in line with the results obtained for the subgenomic HEV genotype 3, the M03.13 cell line supported viral replication as efficient as HepG2 cells with a 100-fold RNA copy number increase over the RBV control after 72h (Fig. 3B and 3G). The neuroepithelioma cell line SK-N-MC, which was treated with 50 μM of RBV, also displayed high RNA propagation efficiency similar to the human liver cells (Fig. 3D). Moderate RNA replication levels were observed for the glioblastoma multiforme cell line DBRTG (Fig. 3C) and the cerebellar medulloblastoma cell line DAOY (Fig. 3E). Although a few antigen positive cells could be observed for the U-373 MG cell line by means of immunofluorescence, results from the qRT-PCR indicate that these cells do not support efficient viral full-length RNA replication (Fig. 3F). In conclusion, with the exception of the glioblastoma astrocytoma cell line U-373 MG, all other neuronal-derived cell line supported full-length HEV RNA replication.

**Neuronal-derived cell lines support HEV assembly and release**

To investigate whether neuronal-derived cell lines are also capable of assembling and releasing HEV particles, the ORF2-encoded capsid protein was quantified by a specific ELISA in the lysate of transfected cells as well as in the respective cell culture supernatant. Similar to the results obtained by qRT-PCR, in all tested neuronal cell lines with the exception of U-373 MG, HEV capsid accumulated intracellularly over time (Fig. 4). This was even

more prominent in M03.13 cells (about 3 $\log_{10}$ OD difference compared to RBV control) compared to HepG2 cells (about 2 $\log_{10}$ OD difference compared to RBV control) (Fig. 4A and F). DAOY, DBTRG and SK-N-MC showed a lesser accumulation of intracellular capsid protein (about 1 $\log_{10}$ OD difference compared to RBV control) (Fig. 4B, C and D). In accordance with the results, determination of extracellular capsid protein demonstrated an increase over time in the cell culture supernatant which was abrogated by RBV treatment (Fig. 5). Here, the HepG2, SK-N-MC and M03.13 cells were equally efficient in releasing capsid (about 2 $\log_{10}$ OD difference compared to RBV control) (Fig. 5A, C and F), which was moderate in DBTRG and DAOY cells (up to 1 $\log_{10}$ OD difference compared to RBV control) (Fig. 5B and D). As expected from the intracellular antigen-content, the U-373 MG cells did not release any capsid in the supernatant (Fig. 5E). Together, intra- and extracellular production of the viral capsid protein as marker for viral assembly and release was detectable in different neuronal cell lines.

**Infection of neuronal-derived cell lines with HEV**

In order to assess the ability of these neuronal cell lines to support HEV entry and RNA replication, we performed an infection assay with cell-culture derived HEV originating from the genotype 3 strain Kernow-C1 p6 as previously described (26). Next to HepG2/C3A cells, a clonal derivate of HepG2 cells, which was previously shown to support HEV entry (25, 26), a set of three neuronal cell line including the glioblastoma multiforme cell line DBTRG, the desmoplastic cerebellar meduolloblastoma cell line DAOY and the oligodendrocytic cell line M03.13 were selected (Fig. 6). Following viral infection, RNA levels in the cell culture supernatants were monitored over time in the presence or absence of 50 µM RBV (Fig. 6). For the human hepatoma cell line HepG2/C3A, an increase in viral titers for over 16 days was noted and RBV treatment blocked this virus production (Fig. 6A). In case of the DBTRG cell line, no significant infection over the RBV control could be observed (Fig. 6B) and for the

DAOY cells even a decrease of viral titers occurred (Fig. 6C). In contrast to these neuronal-derived cell lines, the oligodendrocyte cell line M03.13, which showed the highest capacities for subgenomic and full-length RNA replication as well as capsid assembly and release, demonstrated viral titers accumulating over time. RBV treatment abrogated infectious viral yields showing about 30-fold lower RNA levels in the cell culture supernatant (Fig. 6D). In summary, the oligodendrocytic cell line M03.13 supported productive infection and replication of HEV.

**Discussion**

One of the most frequent extra-hepatic features of hepatitis E virus infection includes a variety of neurological disorders (13). However, so far the underlying virological and pathogenic mechanisms remain unclear. In this study, we characterized the ability of different neuronal-derived cell lines to facilitate the viral life cycle of HEV to dissect neurological extra-hepatic manifestations that have been described to occur in HEV-infected patients (13). By the detection of HEV RNA in the CSF of some HEV patients extra-hepatic reservoirs of HEV have been postulated (15, 21, 22). The results here demonstrated RNA replication in a variety of different neuronal cell lines, especially with the genotype 3-based subgenomic HEV replicon (Fig 1 and 2) supporting the recent suggestion of existing neurotropic HEV variants *in vivo*. Although replication levels of genotype 3 and 1 were similar in the HepG2 liver cells, higher replication levels of the genotype 3 constructs were seen in the DBTRG, SK-N-MC, DAOY and U-373 MG cell lines indicating a genotype-dependent effect for extra-hepatic replication efficiencies. Interestingly, neurological disorders have mainly been described for HEV genotype 3 infections (13), while for genotype 1 and 4 only two patients have been described so far (19, 20). Nevertheless, it has been hypothesized that HEV-associated neurological disorders in Asia are caused by genotype 1 infections, but so far comprehensive clinical studies including the determination of genotypes are missing (13). This HEV genotype-specific observation is further supported by a recent retrospective analysis from India demonstrating that out of 42 patients with neurological symptoms HEV genotype 1 seemed to be a rare cause of neurological syndromes (29). Full-length genotype 3 RNA was used to further examine the complete viral life cycle of HEV in neuronal-derived cell lines (Fig 3-5). Except for the U-373 MG cell line, all tested cell lines also supported full-length HEV RNA replication and showed an increase in intra- and extracellular capsid levels implicating that nervous system-derived cell lines also facilitate viral assembly and release. The oligodendrocytic cell line M03.13, which express phenotypic characteristics of primary

oligodendrocytes (30), showed RNA replication levels comparable to those observed in the liver cell line for the subgenomic replicon as well as in the full-length HEV RNA set up. These nervous system-derived cells also supported efficiently infection with genotype 3 particles, which was not the case for the DAOY and DBRTG cell lines (Fig. 6). RBV was shown to be an effective inhibitor of HEV propagation, which is important to mention as standardized treatment options of HEV-associated neurological symptoms have not been established.

Oligodendrocytes are important for the development of central myelin and the main neurological disorder accompanied by HEV infection is the GBS, which is associated with myelin damage of peripheral neurons (31). GBS has also been described to develop in patients after infection with bacteria like *Campylobacter jejuni* or certain viruses and, in rare cases, after vaccination (32). Therefore, damage of peripheral nerves was also postulated to occur in such infections that elicit an immune response cross-reacting with axolemmal or Schwann cell antigens (31). The completion of the viral life cycle and high replication capacity of HEV in oligodendrocytes observed here might explain the development of GBS syndromes in HEV patients although in the case of GBS the peripheral myelin sheath of the nervous system build up by Schwann cells is affected. Interestingly, the genotype 3 Kernow-C1 p6 strain was originally isolated and subsequently adapted in tissue culture from a patient experiencing chronic hepatitis E with neurological manifestations in terms of peripheral sensory neuropathy and the CSF was tested positive for HEV RNA (15, 25). Notably, also other cases of peripheral neuropathologies in HEV-infected patients have been described in combination with the detection of HEV RNA in the CSF (13), but the potential relationships remain unclear. There is also evidence for a wider range of HEV tissue tropism and extra-hepatic replication from a number of HEV animal models. In genotype 3 infected wild boars HEV RNA was detected in numerous organs including colon, spleen, muscle, uterus and brain (33). Recently, rabbits were experimentally HEV-infected with a trend to chronic infection and

positive/negative RNA as well as HEV antigen expression was observed in several organs including the brain (34). Possible pathological mechanisms of these extra-hepatic viral associations, however, have not been addressed and are difficult to directly compare with the disease in HEV-infected patients. One limitation of our study is that we did not have access to primary human nervous system-derived cells of HEV-infected patients. Post-mortem staining of HEV RNA and/or protein in patients with neurological manifestations may be one way to confirm viral replication in neuronal-derived tissue *in vivo* in futures studies.

In summary, the discovery that HEV is capable of replicating, assembling and entering in specific nervous system-derived cells is the first observations of HEV neuronal tropism and may provide an explanation for the observed neurological symptoms in patients with HEV infection.

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**References**


1. http://www.ictvonline.org.

2. Cao D, Meng XJ. Molecular biology and replication of hepatitis E virus. Emerg Microbes Infect. 2012 Aug;1(8):e17.

3. Behrendt P, Steinmann E, Manns MP, Wedemeyer H. The impact of hepatitis E in the liver transplant setting. J Hepatol. 2014 Dec;61(6):1418-29.

4. Sayed IM, Vercouter AS, Abdelwahab SF, Vercauteren K, Meuleman P. Is HEV an emerging problem in industrialized countries? Hepatology. 2015 Jul 14.

5. Wedemeyer H, Pischke S, Manns MP. Pathogenesis and treatment of hepatitis e virus infection. Gastroenterology. 2012 May;142(6):1388-97 e1.

6. Kamar N, Dalton HR, Abravanel F, Izopet J. Hepatitis E virus infection. Clin Microbiol Rev. 2014 Jan;27(1):116-38.

7. Kamar N, Garrouste C, Haagsma EB, Garrigue V, Pischke S, Chauvet C, et al. Factors associated with chronic hepatitis in patients with hepatitis E virus infection who have received solid organ transplants. Gastroenterology. 2011;140(5):1481-9.

8. Kamar N, Izopet J, Tripon S, Bismuth M, Hillaire S, Dumortier J, et al. Ribavirin for chronic hepatitis E virus infection in transplant recipients. The New England journal of medicine. 2014;370(12):1111-20.

9. Pischke S, Stiefel P, Franz B, Bremer B, Suneetha PV, Heim A, et al. Chronic hepatitis e in heart transplant recipients. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2012;12(11):3128-33.

10. Gerolami R, Borentain P, Raissouni F, Motte A, Solas C, Colson P. Treatment of severe acute hepatitis E by ribavirin. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2011;52(1):60-2.

11. Pischke S, Hardtke S, Bode U, Birkner S, Chatzikyrkou C, Kauffmann W, et al. Ribavirin treatment of acute and chronic hepatitis E: a single-centre experience. Liver international : official journal of the International Association for the Study of the Liver. 2013;33(5):722-6.

12. Zhu FC, Zhang J, Zhang XF, Zhou C, Wang ZZ, Huang SJ, et al. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. Lancet (London, England). 2010;376(9744):895-902.

13. Bazerbachi F, Haffar S, Garg SK, Lake JR. Extra-hepatic manifestations associated with hepatitis E virus infection: a comprehensive review of the literature. Gastroenterol Rep (Oxf). 2015 Sep 10.

14. Dartevel A, Colombe B, Bosseray A, Larrat S, Sarrot-Reynauld F, Belbezier A, et al. Hepatitis E and neuralgic amyotrophy: Five cases and review of literature. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2015;69:156-64.

15. Kamar N, Bendall RP, Peron JM, Cintas P, Prudhomme L, Mansuy JM, et al. Hepatitis E virus and neurologic disorders. Emerging infectious diseases. 2011;17(2):173-9.

16. Woolson KL, Forbes A, Vine L, Beynon L, McElhinney L, Panayi V, et al. Extra-hepatic manifestations of autochthonous hepatitis E infection. Alimentary Pharmacology & Therapeutics. 2014;40(11-12):1282-91.

17. van den Berg B, van der Eijk AA, Pas SD, Hunter JG, Madden RG, Tio-Gillen AP, et al. Guillain-Barre syndrome associated with preceding hepatitis E virus infection. Neurology. 2014 Feb 11;82(6):491-7.

18. van Eijk JJ, Madden RG, van der Eijk AA, Hunter JG, Reimerink JH, Bendall RP, et al. Neuralgic amyotrophy and hepatitis E virus infection. Neurology. 2014 Feb 11;82(6):498-503.

19. Geurtsvankessel CH, Islam Z, Mohammad QD, Jacobs BC, Endtz HP, Osterhaus AD. Hepatitis E and Guillain-Barre syndrome. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2013;57(9):1369-70.

20. Yazaki Y, Sugawara K, Honda M, Ohnishi H, Nagashima S, Takahashi M, et al. Characteristics of 20 Patients with Autochthonous Acute Hepatitis E in Hokkaido, Japan: First Report of Bilateral Facial Palsy Following the Infection with Genotype 4 Hepatitis E Virus. Tohoku J Exp Med. 2015;236(4):263-71.

21. Despierres LA, Kaphan E, Attarian S, Cohen-Bacrie S, Pelletier J, Pouget J, et al. Neurologic disorders and hepatitis E, France, 2010. Emerging infectious diseases. 2011;17(8):1510-2.

22. Kamar N, Izopet J, Cintas P, Garrouste C, Uro-Coste E, Cointault O, et al. Hepatitis E virus-induced neurological symptoms in a kidney-transplant patient with chronic hepatitis. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2010;10(5):1321-4.

23. Nguyen HT, Shukla P, Torian U, Faulk K, Emerson SU. Hepatitis E virus genotype 1 infection of swine kidney cells in vitro is inhibited at multiple levels. Journal of virology. 2014;88(2):868-77.

24. Shukla P, Nguyen HT, Faulk K, Mather K, Torian U, Engle RE, et al. Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. Journal of virology. 2012;86(10):5697-707.

25. Shukla P, Nguyen HT, Torian U, Engle RE, Faulk K, Dalton HR, et al. Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(6):2438-43.

26. Debing Y, Emerson SU, Wang Y, Pan Q, Balzarini J, Dallmeier K, et al. Ribavirin inhibits in vitro hepatitis E virus replication through depletion of cellular GTP pools and is

moderately synergistic with alpha interferon. Antimicrobial Agents and Chemotherapy. 2014;58(1):267-73.

27. Koutsoudakis G, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietschmann T, et al. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. Journal of virology. 2006;80(11):5308-20.

28. Zhou X, Wang Y, Metselaar HJ, Janssen HL, Peppelenbosch MP, Pan Q. Rapamycin and everolimus facilitate hepatitis E virus replication: revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. Journal of hepatology. 2014;61(4):746-54.

29. Goel A, Gupta N, Kalita J, Munjal S, Ranjan A, Misra U, et al. Hepatitis E virus unlikely to be the cause of neurological disease in a genotype 1 endemic region. Journal of viral hepatitis. 2015;22.

30. McLaurin J, Trudel GC, Shaw IT, Antel JP, Cashman NR. A human glial hybrid cell line differentially expressing genes subserving oligodendrocyte and astrocyte phenotype. J Neurobiol. 1995 Feb;26(2):283-93.

31. Hughes RA, Cornblath DR. Guillain-Barre syndrome. Lancet. 2005 Nov 5;366(9497):1653-66.

32. Lehmann HC, Hartung HP, Kieseier BC, Hughes RA. Guillain-Barre syndrome after exposure to influenza virus. Lancet Infect Dis. 2010 Sep;10(9):643-51.

33. Schlosser J, Eiden M, Vina-Rodriguez A, Fast C, Dremsek P, Lange E, et al. Natural and experimental hepatitis E virus genotype 3-infection in European wild boar is transmissible to domestic pigs. Veterinary research. 2014;45:121-014-0121-8.

34. Han J, Lei Y, Liu L, Liu P, Xia J, Zhang Y, et al. SPF rabbits infected with rabbit hepatitis E virus isolate experimentally showing the chronicity of hepatitis. PLoS One. 2014;9(6):e99861.

**Figure legends**

**Figure 1: Replication of subgenomic HEV genotype 3 in different neuronal cell lines**. Different cell lines (A) HepG2, (B) DBTRG, (C) SK-N-MC, (D) DAOY, (E) U-373 MG and (F) M03.13 were transfected via electroporation with HEV RNA of genotype 3 strain Kernow-C1 p6/luc and were left untreated (control) or treated with 100 µM ribavirin (RBV). At 4, 24, 48 and 72 hours post transfection (p.t.), small aliquots of supernatant were removed and viral replication was determined by *Gaussia* luciferase activity. Depicted are the mean values of three independent experiments ± standard error of mean (SEM).

**Figure 2: Replication of subgenomic HEV genotype 1 in different neuronal cell lines**. Indicated cell lines (A) HepG2, (B) DBTRG, (C) SK-N-MC, (D) DAOY, (E) U-373 MG and (F) M03.13 were transfected via electroporation with HEV RNA of genotype 1 strain Sar55 pSK-E2/S17/luc and were left untreated (control) or treated with 100 µM ribavirin (RBV). At 4, 24, 48 and 72 hours post transfection (p.t.), small aliquots of supernatant were removed and viral replication was determined by *Gaussia* luciferase activity. Depicted are the mean values of three independent experiments ± SEM.

**Figure 3: Replication of full-length HEV genotype 3 in different neuronal cell lines.** Different cell lines were transfected via electroporation with HEV RNA of genotype 3 and were left untreated (control) or treated with 50 µM (SK-N-MC) or 100 µM (all other cell lines) ribavirin (RBV). **A)** Immunofluorescence detection of HEV antigen in paraformaldehyde fixed cells. Different cell lines were stained 48 h post transfection (p. t.) for ORF2-encoded protein (green) and the cell nuclei were stained with DAPI (blue). **B)** Cell lysates of lines (B) HepG2, (C) DBTRG, (D) SK-N-MC, (E) DAOY, (F) U-373 MG and (G) M03.13 were analyzed for intracellular RNA at 4, 24, 48 and 72, 96 hours post transfection (h

p.t.) by qRT-PCR targeting the ORF1 gene. Depicted are the mean values of two independent experiments+ SEM.

**Figure 4: Intracellular ORF2-encoded protein levels of HEV genotype 3 in different neuronal cell lines.** Different cell lines (A) HepG2, (B) DBTRG, (C) SK-N-MC, (D) DAOY, (E) U-373 MG and (F) M03.13 were transfected via electroporation with HEV RNA of genotype 3 and were left untreated (control) or treated with 50 µM (SK-N-MC) or 100 µM (all other cell lines) ribavirin (RBV). A commercially available HEV antigen ELISA detecting the ORF2-encoded capsid protein was used to determine intracellular capsid protein amounts 4, 24, 48, 72 and 96 hours post transfection (h p.t.). Depicted are the mean values of two independent experiments + SEM.

**Figure 5: Extracellular ORF2-encoded protein levels of HEV genotype 3 in different neuronal cell lines.** Different cell lines (A) HepG2, (B) DBTRG, (C) SK-N-MC, (D) DAOY, (E) U-373 MG and (F) M03.13 were transfected via electroporation with HEV RNA of genotype 3 and were left untreated (control) or treated with 50 µM (SK-N-MC) or 100 µM (all other cell lines) RBV (RBV). A commercially available HEV antigen ELISA detecting the ORF2-encoded capsid protein was used to determine extracellular capsid protein amount 4, 24, 48 and 72, 96 hours post transfection (h p.t.) in the cell culture supernatant. Depicted are the mean values of two independent experiments ± SEM.

**Figure 6: Infection of different neuronal cell lines with HEV genotype 3**. HepG2/C3A (A) and three selected neuronal cell lines (B) DBTRG, (C) DAOY and M03.13 (D) were infected with a virus stock originating from genotype 3 p6 strain and were left untreated (virus control (VC)) or incubated with 50 µM ribavirin (RBV). Every 2-3 days post infection (d p. i.)

aliquots of culture medium were removed and viral RNA was detected by qRT-PCR targeting the ORF3 region. Depicted are the mean values of three independent experiments ± SEM.

**Suppl. Table 1: Neuronal cell lines used in this study to analyze extra-hepatic replication of HEV.** Overall five different human neuronal cell lines were analyzed for extra-hepatic replication of HEV. The hepatocellular carcinoma cell line HepG2(/C3A) served as a positive control. The following neuronal cell lines were included: glioblastoma multiforme cell line DBTRG, glioblastoma astrocytoma cell line U-373 MG, neuroepithelioma cell line SK-N-MC, desmoplastic cerebellar meduolloblastoma cell line DAOY and the oligodendrocytic hybrid cell line M03.13.