Human liver chimeric mice as a novel model for the study of hepatitis E virus infections.

Alternative titles:

Evaluation and characterization of genotype 1 and 3 hepatitis E virus infections in human liver chimeric mice.

Evaluation and characterization of hepatitis E virus infections of genotype 1 and 3 in mice with humanized liver.

Any other suggestions are welcome

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Running title: HEV infection in humanized mice

Keywords: HEV, humanized mice, genotype, gene expression, ribavirin therapy.

Abstract (max. 250 words; currently 250)

The hepatitis E virus (HEV) is responsible for approximately 20 million infections per year worldwide. Although most infected people can spontaneously clear an HEV infection, patients under immune compromising conditions may evolve to chronicity. Chronic HEV infection can be cured using ribavirin but recently viral isolates with low ribavirin sensitivity have been identified.

Although some HEV isolates can be cultured *in vitro*, *in vivo* studies are essentially limited to primates and pigs. Since the use of these animals is hampered by financial, practical and/or ethical concerns, we evaluated if human-liver chimeric mice could serve as an alternative.

Humanized mice were inoculated with different HEV-containing preparations. Chronic HEV infection was observed after intrasplenic injection of cell culture-derived HEV, a filtered chimpanzee stool suspension and a patient-derived stool suspension. The viral load was significantly higher in the stool compared to the plasma. Overall the viral titer in genotype 3 infected mice was lower than that in genotype 1 infected mice. Analysis of the liver showed the presence of viral RNA and protein, and indicated influenced host gene expression. Intrasplenic injection of HEV-positive patient plasma and oral inoculation of a filtered stool suspension did not result in robust infection. Finally, we validated our model for the evaluation of novel antiviral compounds against HEV using ribavirin.

In conclusion, human liver chimeric mice can be infected with HEV of different genotypes. This small animal model will be a valuable tool for the *in vivo* study of HEV infection and the evaluation of novel antiviral molecules.

Introduction

The hepatitis E virus (HEV) is a small icosahedral virus with a positive sense single stranded (ss) RNA genome of about 7.2 Kb which comprises three open reading frames (ORF1, 2 and 3). ORF1, located at 5' region of the viral genome, encodes a polyprotein with domains with methyl transferase, putative cysteine protease, helicase and RNA-dependent RNA polymerase activity (1, 2). ORF2 is located mainly at the 3' region of the genome and encodes the viral capsid. ORF3, which overlaps with both ORF1 and ORF2, encodes a cytoskeleton-associated phosphoprotein that is involved in virion morphogenesis and egress of virus from cells.

HEV is an enterically transmitted virus that belongs to the *Hepeviridae* family (1). Viruses that can infect humans are classified into genotype (gt) 1 to 4 of the 7 genotypes of the *Orthohepevirus A* genus. Every year approximately 20 million people become infected with HEV worldwide. Most infections occur in developing countries and are mainly caused by gt 1 and gt2 viruses. Overall mortality rates range from 0.5% to 4% but increased mortality is observed in young infants under 2 years of age and peak in pregnant women that are in their third trimester (10 to 20%) (1).

Nevertheless HEV, especially genotype 3, seems to be emerging in the Western world (3). Most infected individuals will spontaneously clear the infection without or with only limited symptoms. Only people under immune compromising conditions (organ transplant recipients, HIV patients, leukemia patients) may evolve to chronicity and are at risk of developing severe liver disease and cirrhosis. Chronic HEV infection can be cured using ribavirin but recently viral isolates with lower ribavirin sensitivity have been identified (4), necessitating the discovery of novel antiviral molecules. The study of HEV replication has been hampered due to the lack of an efficient cell culture system. Recent reports show that certain HEV isolates, mainly of gt3 and gt4, were adapted to grow *in vitro* in cell lines such as HepG2/C3A, PLC/PRF/5 and A549 (5, 6). However, genotype 1 viruses that account for most HEV infection worldwide only poorly replicate in cell culture (7).

Macaques and chimpanzees are the only animals susceptible to infections of all HEV genotypes (8, 9), while pigs can be used for propagation of HEV gt3 and gt4 only (10, 11). Until now, a small animal model for the study of HEV is still lacking. Here we report on the evaluation and characterization of HEV infection in human liver-chimeric mice. These mice are generated by transplanting primary human hepatocytes into immune deficient uPA-SCID mice that suffer from a transgene-induced liver disease. Several weeks after transplantation, the major part of the mouse liver is repopulated by functional human hepatocytes, which renders these chimeric mice susceptible to human-specific hepatotropic pathogens such as hepatitis B virus, hepatitis C virus and *Plasmodium falciparum* (12-15). Our data show that these humanized mice are also susceptible to HEV infection and that they can be used for the preclinical *in vivo* evaluation of novel antiviral compounds.

Materials and Methods

Production of cell culture HEV (HEVcc).

HEV Kernow-C1 Passage 6 (P6) plasmid (gt3) (5) was digested by MluI enzyme and capped RNA was produced using T7 RiboMAX Large Scale RNA production (Promega, Belgium) and ScriptCap m7G capping system (CellsScript, Madison, USA). Transfection of HepG2/C3A cells in Dulbecco's modified Eagle's medium (DMEM) was done using lipofectamine 2000 (Life technologies, Belgium). Culture medium was collected at different time points and tested for the presence of viral RNA by qPCR, as described below. Certain culture supernatant (SN) aliquots were treated (TSN) with 0.1% trypsin and 0.1% sodium deoxycholic acid at 37°C for 1 hour to mimic the process that HEV particles undergo when excreted in the feces (6, 16). Lysis of transfected cells to release intracellular viral particles (cell lysate) was done by vigorous vortexing in the presence of high salt concentration (17). The infectivity of HEVcc was tested on naïve HepG2/C3A cells.

Production, infection and treatment of human liver chimeric mice.

Approximately 10^6 primary human hepatocytes (donor HH223, Corning, The Netherlands) were transplanted into homozygous uPA^{+/+}-SCID mice as described previously (12, 18, 19). Human albumin, determined by ELISA (Bethyl Laboratories Inc, Montgomery, USA), was used as marker to assess liver chimerism.

Humanized mice were inoculated with different HEV preparations, mainly of gt3 (obtained from an infected patient and HEVcc) and gt1 (strain Sar-55; stool suspension obtained from an infected chimpanzee (9)). Stool and plasma samples were collected on a weekly basis. Detection and quantification of viral RNA in mouse plasma and 10% (w/v) mouse stool was done using RT-qPCR (see below). Non-transplanted mice inoculated with these preparations served as negative control. Passage experiments were performed by inoculating naïve humanized mice with EDTA plasma or a 10% (w/v) 0.22µm filtered stool suspension obtained from a Sar-55 infected humanized mouse. Inoculations were performed via oral and intrasplenic route.

Ribavirin (RBV) was dissolved in saline solution and administered orally at a single daily dose of 25, 50 or 100 mg/kg. Control mice received vehicle only.

Quantification of HEV RNA.

HEV RNA levels were quantified via RT-qPCR using primers (5'-GGTGGTTTCTGGGGTGAC-3' (F) and 5'-AGGGGTGGTTGGATGAA-3' (R)) and a probe (5'-FAM-TGATTCTCAGCCCTTCGC-TAMRA-3') that target a conserved 70bp-region in the ORF2/3 overlap (20). A standard curve was generated and calibrated against the WHO 1st international standard (Paul-Ehrlich Institute, Germany) (21). HEV RNA was extracted from plasma and feces using the NucliSENS easyMAG system (Biomérieux, France) and a one-step real-time RT-qPCR was performed on the LightCycler480 (Roche, Belgium) using the Roche Lightcycler Multiplex RNA virus master mix. The limit of quantification (LOQ) of our assay on undiluted samples was 40 IU/ml and equals the 100% limit of detection (LOD).

Analysis of liver from infected humanized mice.

Total DNA, RNA and protein were extracted from 30 mg of liver tissue from humanized mice using the Allprep RNA/DNA/protein mini kit (QIAGEN, The Netherlands) according to manufacturer instructions. HEV RNA was quantified in total liver RNA by RT-qPCR as detailed above. HEV RNA was quantified in 3 different liver fragments and the mean viral load was calculated.

A previously published technique (22) was adapted for the specific detection of positive and negative-sense HEV RNA of all the HEV genotypes used in our study. Briefly, liver RNA extract was converted into cDNA using Superscript III enzyme (Life Technologies, Belgium) with primers specific for either positive or negative-sense RNA (Table 1). After treatment with Exonuclease I (Life Technologies, Belgium) and purification using the Wizard SV gel and PCR Clean-Up (Promega, Benelux), purified cDNA was used as template for nested PCR with the primers shown in Table 1. *In vitro* transcribed RNA corresponding to negative-strand RNA was produced and served as positive control for the negative-strand PCR and as negative control for the positive-strand PCR. Stool of infected mice served as positive control for the positive-strand PCR and as negative control for the negative-strand PCR. Extracts from HEV-infected HepG2/C3A were used as positive control for both positive and negative-strand specific PCRs. Liver extracts were diluted such that the viral RNA load was lower than that in the fecal control samples, thereby ensuring that a signal representing negative-strand RNA was not caused by aspecific amplification of excess positive-strand viral RNA.

For Western blot analysis equal amounts of purified protein were loaded and run on a 10% SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane, which was incubated overnight at 4° C with a rabbit polyclonal antibody against HEV ORF3 (clone x; 1/1000). Lysate from infected cell cultures and protein extracts from non-infected humanized mouse liver served as positive and negative controls. Immunofluorescence analysis was performed on 4% paraformaldehyde-fixated chimeric mouse livers using an ORF3-specific rabbit polyclonal antibody (original source?). Livers from non-infected humanized mice, livers from non-transplanted mice inoculated with the same HEV preparations and livers from humanized mice inoculated with other hepatotropic pathogens (HBV, HCV and malaria) were processed in the same way and served as negative control.

Host gene expression analysis.

Total liver RNA and DNA were extracted from scarified HEV infected mice (gt1, Sar-55) (n=2) as described above. DNA was used to confirm humanization using the qPCR assays described previously (13). The presence of HEV RNA was confirmed using the RT-qPCR assay described above. RealTime Assay 96 well plates precoated with 48 selected primer-probe pairs were obtained from Roche (Zaventem, Belgium). RNA from infected livers was reverse transcribed using the Superscript III kit (Life Technologies, Belgium). cDNA was then added to each well on the plate (two tissue samples per plate) at a concentration of 25ng equivalent total RNA per reaction following manufactures' protocol. QPCR was run on a LightCycler 480 (Roche, Zaventem, Belgium) and relative quantification was calculated using qbase+ (Biogazelle, Zwijnaarde, Belgium). Total liver RNA and DNA was extracted from a non-infected humanized mouse served as negative control. Three different samples from each mouse were analyzed and the geometrical mean was calculated. As control for mouse cross reactivity, a non-transplanted mouse was injected with 30 µg Poly I:C (Invivogen, Toulouse France) and sacrificed 8 hours later (23). Five house-keeping genes, of

which we selected the 3 most stable ones using geNorm (qbase+ program, Biogazelle), served as reference for normalization of the amount of cDNA that was added into each well.

Results

Infection of humanized mice with cell culture-derived HEV (HEVcc).

HepG2/C3A cultures were transfected with *in vitro* transcribed capped RNA of the gt3 HEV isolate Kernow C1-p6. Cell culture supernatant was harvested and administered to humanized mice. Initial experiments involving oral (n=2) and intrasplenic (n=6) administration of up to 1.1×10^6 IU HEVcc did not result in a detectable infection.

Next we injected HEVcc preparations that underwent sodium deoxycholic acid and trypsin treatment, thereby mimicking the process viral particles undergo after shedding into the bile duct and passage through the duodenum. Although intrasplenic injection of 3.3×10^5 IU/mouse (n=2) did not result in infection, 1 out of 3 mice inoculated with 3.2×10^6 IU/mouse showed signs of active infection (Figure 1a). HEV RNA was only detectable in 10% stool suspensions starting from week 3 until week 9 post inoculation, after which the viral titer decreased.

Finally, HEV RNA could be detected in the stool of 2 out of 3 humanized mice injected with lysate from an infected cell culture (1.7×10^6 IU/mouse). Virus was detected from week 1 on and titers steadily increased reaching levels up to 6.2×10^4 IU/ml (Figure 1b).

HEV RNA could not be quantified in the plasma of any of the infected humanized mice, nor in the plasma or feces of inoculated non-humanized negative control mice.

Infection of humanized mice with chimpanzee-derived HEV.

A 10% stool suspension obtained from a chimpanzee infected with HEV strain Sar-55 was used as inoculum for genotype 1 infection studies. Two humanized mice that were intrasplenically inoculated with 3×10^6 IU/mouse became robustly infected. HEV RNA could be detected both in the feces and plasma starting from week 1 post inoculation and reached levels exceeding 10^7 IU/ml and 10^6 IU/ml respectively (Figure 2a). Viral titers in stool suspensions were in general about 10 to 100-fold higher than those in plasma. HEV RNA could not be detected in stool and plasma of non-transplanted mice inoculated with the same preparation.

A 10-fold lower dose of chimpanzee derived Sar-55 (3×10^5 IU/mouse) also resulted in infection of all inoculated humanized mice (n=5) (Figure 2b). HEV RNA could be detected in the stool of all mice from week 1 post inoculation and viral titers sharply increased over time indicating robust infection. HEV RNA could initially only be detected in plasma of 3 mice from week 2 on, but became detectable in the remaining two mice at week 5 and 6 respectively (Figure 2b). Overall there was a considerable delay in the kinetics of the viral infection compared to the mice that received a 10-fold higher inoculum dose.

To investigate the infectious nature of the viral particles identified in humanized mouse feces we prepared a 10% filtered stool suspension that was subsequently used as inoculum to infect naive humanized mice. All mice (n=3) inoculated with 2.28×10^5 IU Sar-55 became infected (Figure 3a). HEV RNA could be detected in stool and plasma of all mice from the first week post inoculation and the viral titer increased over time indicating replication of the virus *in vivo*. After 4-5 weeks of infection, HEV titer reached levels up to 1.2×10^7 IU/ml in the stool and ranged from 6.9×10^3 IU/ml to 1.2×10^6 IU/ml in the plasma. One week after inoculation with a mouse Sar-55 fecal suspension, we could detect a minute amount of HEV RNA near the limit of detection of our assay in the feces of one non-humanized control mouse. Since no viral RNA could be detected in all subsequent samples we assume this was either

residual input RNA and/or contamination due to cohousing of this mouse with the 3 other humanized mice that already exhibited a high level of infection at that time point (Figure 3a).

A humanized mouse was also intrasplenically inoculated with plasma obtained from an HEV-infected mouse. One week after inoculation (10^5 IU HEV RNA) the feces scored HEV RNA negative ($<4 \times 10^2$ IU/ml), but at week 2 and 3 we obtained a specific signal corresponding to respectively $3,98 \times 10^3$ and $4,6 \times 10^3$ IU/ml. This mouse died a few days later so further follow-up was not possible.

To determine the minimal infectious dose of our Sar-55 preparation in humanized mice we performed additional infection experiments. Two mice were challenged IS with mouse stool containing 2.5×10^4 IU/mouse and readily became infected. HEV RNA could be detected in the stool of both mice from week 1 post infection, while viremia was delayed until week 3 post infection. The stool viral titer of this group was lower than the titer observed in the previous 3 mice, probably due to lower inoculum dose (Figure 3b).

A further decrease in inoculum dose, 1.2×10^3 IU/mouse, resulted in only 1 out of 2 mice becoming infected. In the infected mouse, HEV RNA could be detected in stool but the titer was relatively low (up to 10^4 IU/ml) (Figure 3c). Viremia could only be detected at week 5 post inoculation. The second mouse died 3 weeks after inoculation but we could not detect HEV RNA in the stool during this period (Figure 4c).

Finally three humanized mice received an oral inoculation at a dose that was previously shown to be 100% infectious via intrasplenic route (2.28×10^5 IU/mouse) but in none of them HEV RNA became detectable during the 8 week observation period. Interestingly, IS inoculation of a lower dose (2.5×10^4 IU/mouse) into one of the unsuccessfully orally challenged mice resulted into infection, indicating that IS inoculation is much more efficient than oral gavage in establishing HEV infection in humanized mice.

Infection of humanized mice with patient stool and plasma.

To investigate whether humanized mice could also be infected with patient-derived HEV of genotype 3 we inoculated two chimeric mice with a human stool suspension containing 3.95×10^5 IU/mouse. Both mice became infected as evidenced by the presence and increase of viral RNA in fecal suspensions. While the viral load in stool ultimately reached levels at around 10^5 IU/ml, HEV RNA could only be detected in the plasma of one of the two mice and at a later time point (Figure 2c). Non-humanized mice injected with the patient stool suspension and a single highly humanized mouse inoculated with plasma obtained from the same patient (dose 6.5×10^4 IU/mouse) never showed any signs of viral infection.

Detection of viral RNA and protein in the liver of infected humanized mice.

Total RNA extracts were prepared from the liver of mice infected with either HEV of gt1 strain Sar-55 (n=2), patient-derived HEV of gt3f (n=1) or HEV produced in cell culture (n=1). The mean HEV RNA load in a 30 mg-fragment was respectively 1.31×10^7 IU, 1.03×10^7 IU, 6.88×10^5 IU and 1.47×10^4 IU. These levels correlate with the viral titer in stool and plasma of these infected mice and indicate a higher level of viral replication of gt1 virus compared to that of gt3 viruses.

Using strand-specific PCR we were able to specifically show the presence of replicative-intermediate negative-strand HEV RNA in the livers of infected humanized mice, but not in corresponding fecal

samples (Figure 4). Semi-quantitative analysis indicated that the (+)/(-) strand RNA ratio was at least 1,000 to 1 (data not shown).

Detection of viral protein in the liver of infected humanized mice.

Paraffin-embedded liver tissue sections from HEV infected humanized mice, non-infected mice and mice infected with other hepatotropic pathogens (HBV, HCV and malaria) were stained with a polyclonal antibody against HEV-ORF3. A clear and strong signal was detected specifically in the bile canicular network formed by the human hepatocytes inside infected mouse liver (Figure 5). Areas occupied by mouse hepatocytes stained completely negative, as did livers from non-infected mice and mice infected with other pathogens. The intensity of ORF3 staining in the liver correlated with the viral titer determined at time of sacrifice. The presence of ORF3 in infected livers was confirmed using Western blotting (Figure 6).

HEV influences host genome expression.

To evaluate whether HEV infection has an influence on the gene expression of the infected hepatocyte, we analyzed liver tissue of an uninfected mouse and compared the gene expression profile with that of two animals that were infected for 4 and 9 weeks with gt1 Sar-55. Our analysis was restricted to genes involved in the immune response and only human-specific primer/probe sets were used that did not cross-react with mouse genes. We observed a considerable increase of CXCL9 and CXCL10 (respectively 23,7 and 8,5-fold at week 9), two chemokine receptor ligands involved in leukocyte stimulation and trafficking, and adhesion molecule expression (Table 2). A 2- to 3-fold increase of HLA-A, B, F and J was observed after 9 weeks of infections. In addition, several other interferon-induced genes were upregulated, such as IFI27, IFI44L, IFIT1, IFI2, IFIT3, ISG20, OAS2, OASL, RSAD2, TAP1 and TRIM22 (Table 2). Overall we observed a more pronounced effect at week 9 of infection compared to week 4. A variety of genes involved in the innate immune response were not affected by the infection. These include USP18, TLR3, ISG15, STAT1, STAT2, OAS1, IRAK1, MX1, MAVS, etc. (Table 2).

Ribavirin treatment of HEV infected mice.

Finally we wanted to validate our mouse model for the evaluation of novel antiviral compounds. Since ribavirin (RBV) is currently the first choice therapy for chronic HEV infection we used this molecule for antiviral therapy of infected chimeric mice.

We started a dose-escalation experiment in two infected chimeric mice. One mouse was used as control and received vehicle only while the second one first received a 1-week oral RBV treatment of 25 mg/kg qd. As shown in Figure 7a, a slight decrease in stool viral titer was observed ($-0.51 \times \log_{10}$). Although an additional 2-week treatment at 50 mg/kg did have a major effect, a further increase to 100 mg/kg resulted in a sharp $1.60 \times \log_{10}$ drop in viral titer within 6 days. However, since this dose was not well tolerated we decided to treat a larger mouse cohort with a daily RBV dose of 50 mg/kg.

A total of 5 HEV infected humanized mice started a 2-week therapy RBV therapy (Figure 7b). One mouse died after 7 days of treatment while a second one died after 12 days. In the latter we observed a $0.66 \times \log_{10}$ reduction in fecal viral titer compared to baseline. While the fecal viral titer in two control mice remained stable (average change of -0.13 and $+0.21 \times \log_{10}$ IU/ml after 7 and 14 days respectively), a

more than 10-fold decrease ($n=4$) was observed after 10 days (Figure 7c). An average decrease of $-1,79 \times \log_{10}$ IU/ml ($n=3$) was observed at the end of treatment (EOT). In one animal we did not observe a change in fecal viral titer between day 10 and day 14. However, an increase of RBV dose to 100 mg/kg resulted in an additional $-0,72 \times \log_{10}$ IU/ml within 5 days (Figure 7b).

The decrease in fecal viral load coincided with a decline in viremia (Figure 7b). The two animals that died before EOT experienced a $0,78$ to $2,47 \times \log_{10}$ drop in viremia, reaching levels below the limit of quantification. Of the 3 remaining mice, two reached a plasma viral load at or below LOQ at EOT ($-1,29$ and $-3,04 \times \log_{10}$ IU/ml), while the drop in the 3d one was less pronounced ($-0,57 \times \log_{10}$). We also observed a less pronounced ORF-3 staining in the liver of RBV-treated mice (data not shown). Follow-up was performed on two mice that reached EOT. After cessation of therapy, the fecal viral titer respectively increased $1,35$ and $2,19 \times \log_{10}$ -fold, while viremia became detectable but unquantifiable in one mouse and increased at least $1,68 \times \log_{10}$ -fold in the other mouse.

Discussion

The lack of a small animal model and efficient cell culture systems has hampered the study of HEV replication and pathogenesis. Since HEV appears to emerge in industrialized countries and viral isolates with decreased sensitivity to ribavirin become prominent, such models will be of great importance for the identification and pre-clinical evaluation of novel antiviral compounds.

Here we show that human liver chimeric mice are suitable for the study of HEV replication, virus-host interactions and the evaluation of antiviral therapies. To evaluate the susceptibility of humanized mice to HEV infection we evaluated viral preps of different origins. The initial infection studies were performed with cell-culture derived virus of gt3, strain Kernow C-1 P6. In general, only a minority (3/15) of the challenged mice became infected and viral titers in stool were relatively low, while being negative in the plasma. The low viral replication of this particular strain may be explained by its adaptation to grow in cell culture and/or to a recombination event resulting in an insertion of 174 ribonucleotides (58 amino acids) of the human ribosomal protein S17 gene into the viral genome (5). Interestingly, infections were only successful if the mice were challenged with viral particles that were pretreated with trypsin and sodium deoxycholic acid (1/5); or that originated from cell lysates (2/3). This may be related to differences in particle composition between intracellular and secreted virus. It has been reported that secreted viral particles are associated with lipids, while intracellular particles and particles treated with trypsin/sodium deoxycholic acid, which mimics the transformation secreted particles undergo during passage through the bile duct and intestine, are not associated with lipids (6, 16, 24-26). Our data indicate that these differences in particle composition have an impact on viral infectivity. Further studies are needed to ascertain the interplay between particle composition and viral infectivity.

Infections with patient (gt3f) and chimpanzee (gt1, Sar-55) stool suspensions were more successful. All intrasplenic inoculations lead to active infection of the humanized mice, evidenced by the presence and increase over time of HEV RNA in the stool; and viremia in those mice with highest fecal titer. Overall the viral titer was much higher in mouse stool compared to the plasma, which is similar to what has been observed in chimpanzees, cynomolgus monkeys, pigs and humans (27-30). HEV RNA could also be detected much earlier in the stool than in plasma, which is in line with what was observed in experimentally infected chimpanzees (27). The infection was further corroborated by the presence of both positive and negative strand viral RNA and viral protein in the infected mouse liver; and by successful serial passage of the infection from one infected humanized mouse to a naïve one. Western blot analysis indicated that the molecular weight of ORF3 protein present in mouse liver was slightly higher compared to that present in lysate of an infected cell culture. This may be related to differences in posttranslational modifications but further study is needed to corroborate this.

Comparison of peak viral titers between gt1 and gt3 infections indicated much higher replication of the former. This is in agreement with previous publications and clinical data indicating that gt1 viruses are more virulent than gt3 viruses (9, 31, 32). These data also highlight that the low replication rate observed with cell culture-derived virus may not (completely) be related to cell culture adaptation, but may (in part) also be caused by the general characteristics of genotype 3 viruses.

Interestingly we were unable to infect humanized mice using HEV-positive patient plasma. Using a slightly higher dose of HEV-positive mouse-derived plasma, we were able to introduce an HEV infection in a naïve mouse but the kinetics of the infection were much lower to what was observed in other mice

that were inoculated with a similar or lower dose of the same viral strain. This indicates that the infectivity of viral particles in plasma is most likely much lower than that of particles present in feces. It has been reported that fecal viral particles have a higher buoyant density (1.27 to 1.28 g/ml) than HEV particles present in plasma (1.15 to 1.16 g/ml) (26). This difference is probably provoked by the association of lipids to the latter. Further studies are needed to ascertain whether the difference in particle composition, and more specifically the association with lipids, has a negative impact on viral infectivity.

We also attempted to infect humanized mice via the oral route, the presumed natural route of HEV transmission. However, while a single oral dose of 2.28×10^5 IU/mouse was not successful, a 10-fold lower intrasplenic dose was sufficient to establish an infection, indicating that the failure of oral transmission was not related to the level of mouse liver chimerism. Similar findings were reported by Tsarev and colleagues, who showed that oral inoculation of cynomolgus monkeys required at least a 10,000-fold higher dose than IV inoculation to establish an HEV infection (33). Likewise, pigs could be experimentally infected with a single intravenous injection of as little as 10^2 genome equivalents, while multiple high-titrated oral inoculations, containing more than 10^6 genomes, established infection in only one third of the animals (34, 35). These data clearly show that, although it is considered as the natural way of infection, transmission via oral route is not efficient.

We also evaluated the direct influence of HEV infection on gene expression of the infected hepatocyte. For this purpose our humanized mice are a unique tool because they lack a functional adaptive immune system, which otherwise also would influence the host's gene expression profile. Our data show that a large number of ISGs are directly upregulated upon infection. These include chemokines, molecules involved in antigen presentation and several proteins known to negatively influence viral translation and replication.

Finally we validated our chimeric mouse model for the evaluation of novel antiviral therapies. Ribavirin (RBV) is currently the first-choice antiviral treatment in chronically infected HEV patients (36). A therapy of at least 3 months is usually sufficient to eliminate the infection (37, 38). We were able to show a dose-dependent reduction of viral load upon RBV treatment. A 2-week RBV therapy resulted in an average $1,79 \times \log_{10}$ -fold reduction in fecal viral load, which coincided with an even more pronounced reduction in viremia. Viral rebound was observed upon cessation of RBV therapy. At the end of therapy the plasma viral titer was below the limit of detection, while HEV RNA still could be detected in mouse stool. These results are similar to clinical data. Ambrosioni et al. reported that during prolonged RBV therapy of a leukemia patient with chronic HEV, viral RNA became undetectable in the plasma after 6 months, while viral particles were continuously shed in the feces for an additional 6 months (39). In another study, 3 months of RBV therapy of 24 cases of chronic HEV organ transplant patients lead to 100% clearance of the virus from plasma, while HEV RNA could still be detected in stool in 25% (6/24) of cases (38). Viral rebound after cessation of a 3-month RBV therapy was also observed by Abravanel et al. in 9/24 cases (38). At EOT, HEV RNA was still detectable in the stool of 6 out of these 9 patients, while it was undetectable in the plasma.

We here report the establishment and characterization of HEV infection in mice with humanized liver. This model will be an excellent tool for the *in vivo* study of the basic aspects of the HEV life cycle and the

interactions between the virus and its host. Furthermore, this model can be used for the preclinical in vivo evaluation of novel antiviral therapies.

Acknowledgements.

This study was funded by the Ghent University (Concerted Action Grant 01G01712), The Research Foundation – Flanders (FWO-Vlaanderen; project G0D2715N), the Belgian Federal Government (IUAP P7/47-HEPRO-2), and the European Union (FP7, HepaMab). IMS is a recipient of a PhD Fellowship provided by the Egyptian Government. LF was supported by a PhD-fellowship of the Agency for Innovation by Science and Technology (IWT). Yannick Debing received a PhD fellowship from the Research Foundation – Flanders (FWO).

Figure legends.

Figure 1: infection of humanized mice with cell culture derived virus. Human liver chimeric mice were inoculated with (A) supernatant treated with sodium deoxycholic acid and trypsin or (B) lysate of HEV-infected cell cultures (genotype 3, strains Kernow C1-P6). Viral RNA was quantified at different time points in 10% fecal samples (black) and plasma (red). Solid lines represent data obtained from humanized mice while dotted lines relate to data obtained from inoculated non-humanized control mice. (LOQ represents the limit of quantification.)

Figure 2: infection of humanized mice with human or chimpanzee derived HEV preparation. Human liver chimeric mice were inoculated intrasplenically with a stool suspension of an infected chimpanzee containing 3×10^6 IU (A) or 3×10^5 IU (B) HEV RNA (genotype 1, strain Sar-55). An additional group of mice was inoculated with a fecal suspension of a patient infected with HEV of gt3f (C). Viral RNA was quantified at different time points in 10% fecal samples (black) and plasma (red). Solid lines represent data obtained from humanized mice while dotted lines relate to data obtained from inoculated non-humanized control mice. (LOQ represents the limit of quantification.)

Figure 3: HEV Sar-55 serial passage and titration experiments in humanized mice. A 10% stool suspension was prepared from a Sar-55 infected humanized mouse and used to inoculate naïve humanized mice. Each mouse was injected intrasplenically with a suspension containing $2,28 \times 10^5$ (A), $2,5 \times 10^4$ (B) or $1,2 \times 10^3$ (C) IU HEV RNA. Viral RNA was quantified at different time points in 10% fecal samples (black) and plasma (red). Solid lines represent data obtained from humanized mice while dotted lines relate to data obtained from inoculated non-humanized control mice. (LOQ represents the limit of quantification.)

Figure 4: detection of (+) and (-) sense HEV RNA in the liver of infected humanized mice. Plus strand-specific (lane 2-10) and minus strand-specific (lane 11-19) RT-PCR was performed on liver extracts obtained from mice infected with HEV Sar-55 (lane 2 and 11), patient gt3f (lane 3 and 12) or gt3 HEVcc (lane 4 and 13). Liver extracts from non-inoculated humanized mice (lane 5 and 14), a stool sample from a gt1-infected mouse (lane 6 and 15) and a gt3f-infected patient (lane 7 and 16), extracts from gt3-infected HepG2 cells (lane 8 and 17), in vitro transcribed (-) RNA (lane 9 and 18) and non-template control (lane 10 and 19) were included as positive and negative controls. Minus strand HEV RNA could specifically be detected in liver extracts of infected mice and infected cell lysate, while it was absent in corresponding fecal samples. Lane 1 shows the pattern of a 100bp DNA ladder.

Figure 5: visualization of ORF3 protein in HEV infected humanized mouse livers using immunofluorescence. Within the liver of a chimeric mouse, areas repopulated by human hepatocytes can be easily discerned from residual mouse parenchyma by the larger size and paler color of the former (A). Liver sections prepared from a non-infected humanized mouse (B, E) and humanized mice infected with chimpanzee (C, F) or mouse (D, G) -derived Sar-55 were stained with a polyclonal antibody against HEV ORF3 protein. ORF3 could be specifically observed in areas repopulated with human hepatocytes

while mouse areas stained negative. The staining pattern indicates the presence of HEV ORF3 in the bile canalicular network formed by human hepatocytes. (E), (F) and (G) show a higher magnification (000X) of the area indicated with a white square in panels (B), (C) and (D) (000X).

Figure 6: visualization of ORF3 protein in HEV infected humanized mouse livers using Western blot.

Protein extracts from the liver of two Sar-55 infected mice (lane 1 and 2) and one uninfected control mouse (lane 3) were separated on a 10% SPS-PAGE gel and transferred to a nitrocellulose membrane. ORF3 protein was visualized by overnight staining with a polyclonal antibody. A protein extract from an HEV infected cell culture was used a positive control (lane 4).

Figure 7: ribavirin (RBV) treatment of HEV infected mice. (A) A pilot study was performed to determine

the optimal RBV dose. Repetitive dose escalation indicated that the highest response was observed at 100 mg/kg daily. Because this dose was poorly tolerated, 50 mg/kg was used in the subsequent studies. (B) A total of 5 mice were treated with RBV (red lines) and viral RNA was quantified at different time points in the feces (solid line) and blood (dotted line). Two infected mice received vehicle only and served as negative controls (black lines). Upon RBV treatment, a considerable drop in viral load was observed, both in the plasma and feces. In one animal (indicated with a rhombus) the drop in fecal viral titer was much less pronounced although there was a $3 \times \log_{10}$ -fold drop in viremia. An increase in RBV dose to 100 mg/kg (blue line) resulted in a $0,72 \times \log_{10}$ -fold drop in fecal HEV titer within 5 days. Two mice were followed-up after therapy cessation and a rebound in viral titer was observed. (C) Overall, a 14-day RBV treatment at 50 mg/kg resulted in an average drop in fecal viral titer of $1,64 \times \log_{10}$ IU/ml ($n=3$).

Table 1: primers used for specific amplification of plus- or minus-strand HEV RNA.

	(+) strand RNA	(-) strand RNA
cDNA synthesis	5'-CGGTCATGGTGGCGAATAARTGTTATTCATTCYAMCK-3'	5'-CGGTCATGGTGGCGAATAAYGARAAYGAYTTYTCHGAGTTTG-3'
1st round PCR	Forward: 5'-YGARAAYGAYTTYTCHGAGTTTG-3' Reverse: 5'-CGGTCATGGTGGCGAATAA-3'	Forward: 5'-CGGTCATGGTGGCGAATAA-3' Reverse: 5'-RTGTTATTCATTCYAMCK-3'
2nd round PCR	Forward: 5'-AYACYRTYTGGAAAYATGGC-3' Reverse: 5'-ARCATGCCAATMAGGTTATG-3'	Forward: 5'-AYACYRTYTGGAAAYATGGC-3' Reverse: 5'-ARCATGCCAATMAGGTTATG-3'

Y= C+T, R= A+G, H= A+T+C, M=A+C, K=G+T and 5'CGGTCATGGTGGCGAATAA3' is a non-HEV tag sequence used as target for the 1st round PCR.

Table 2: host gene expression analysis of HEV-infected livers.

Upregulated genes ^(a)				Unaffected genes ^(a)	
Gene name	Aliases	Average fold change ^(c)		Gene name	Aliases
		4 weeks of infection	9 weeks of infection		
CxCL-9		5.14	23.66	USP18	ISG43
CxCL10	IP-10, IFI10	3.10	8.54	UBC	
IFI27	ISG12	2.99	4.88	TLR3	CD283
IFI44L		10.23	14.36	ISG15	IFI15, IP17
IFIT1	ISG56	2.83	2.05	STAT1	
IFIT2	ISG54	6.55	4.43	STAT2	
IFIT3	ISG60, RIG-G	2.73	2.72	OAS1	IFI4
RSAD2	Viperin	8.90	5.42	IRAK1	
ISG20	CD25	NA	3.25	MX1	IFI78, MxA
OAS2		2.17	NA	MAVS	CARDIF, IPS1, VISA
OASL	TRIP14	2.86	NA	IRF1	
TAP1		NA	2.53	IRF3	
TRIM22		NA	3.39	IRF7	
HLA-A		NA	2.10	IFITM3	IP15
HLA-B		NA	3.02	IFI44	
HLA-F		NA	2.24	IFI6	
HLA-J		NA	3.09	DDX60	
				HLA-C	

(a) Upregulated genes: fold change ≥ 2 compared to control mice.

(b) Unaffected genes: fold change between 0.5-2 compared to control mice.

(c) Geometric mean of 3 different fragments. NA: not significantly affected (fold change between 0,5-2).

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Figure 1

Figure 2

Figure 3

Fecal HEV titer - Plasma HEV titer

Figure 7