New models of hepatitis E virus replication in human and porcine hepatocyte cell lines

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Hepatitis E virus (HEV) causes acute, enterically transmitted hepatitis in human. It is associated with large epidemics in tropical and subtropical regions where it is endemic or with sporadic cases in non-endemic regions. Unlike other hepatitis viruses, HEV has several animal reservoirs. Phylogenetic studies on HEV human and animal sequences, and the identification of cases of direct transmission from animal to human strongly suggest that HEV is a zoonotic agent. The lack of efficient cell culture models limits studies on molecular and cellular aspects of HEV infection and species barrier crossing. The present study reports on the development of two new in vitro models of HEV replication using a human hepatoma-derived cell line, HepaRG, and a porcine embryonic stem cell-derived cell line, PICM-19. These two cell lines have morphological and functional properties similar to primary hepatocytes. These in vitro culture systems support HEV replication and release of encapsidated RNA. These new models represent a powerful tool for studying the viral replication cycle, species barrier crossing and virulence factors.

INTRODUCTION

Hepatitis E virus (HEV) is responsible for large epidemics of acute hepatitis in developing countries, and for autochthonous sporadic cases in industrialized countries. The evolution of hepatitis E is often benign, but severe forms have been reported. High rates of fulminant hepatitis have been observed in pregnant women (20%) from some endemic regions and in patients suffering from liver conditions caused by hepatitis C virus (HCV) and hepatitis B virus (HBV) infections or by alcohol abuse (Dalton et al., 2011; Khuroo et al., 1981). Chronic HEV infections have been recently described in immunosuppressed patients (Kamar et al., 2010). The mechanisms responsible for the different degrees of severity of hepatitis E infections are still unknown.

HEV is a non-enveloped, single-stranded, positive-sense RNA virus, classified in the genus Hepeviridae of the family Hepeviridae (ICTV, 2009). The genome is 7.2 kb in length and contains three major ORFs. The ORF1 encodes non-structural proteins including methyltransferase, protease, RNA helicase and RNA-dependent RNA polymerase motifs (Ahmad et al., 2011; Ansari et al., 2000; Jameel, 1999; Panda, 2000). The ORF2 and ORF3 overlap and two proteins are translated from a single subgenomic RNA (Graff et al., 2006). The ORF2 encodes the viral capsid protein and the ORF3 protein is a small phosphorylated protein made of 113–123 aa, whose function(s) has not been fully defined (Emerson et al., 2010). HEV replicates in liver cells and virions are secreted into the intestinal lumen via the bile duct and subsequently excreted within the faeces.

HEV sequences can be classified into four major genotypes. Genotypes 1 and 2 have been reported in humans from Asia and Africa, or from Mexico, respectively. Genotypes 3 and 4 have been identified both in humans and in animal species, mostly swine, in industrialized countries as well as in Asia (Purcell & Emerson, 2008).

In developing countries, the main transmission pathway of hepatitis E infection is through consumption of contaminated water or soil传. In contrast, in industrialized countries, contamination pathways are still not clear. Zoonotic transmission, through ingestion of raw or undercooked contaminated deer and boar meat, have been described in sporadic cases of acute hepatitis E in humans (Masuda et al., 2005; Tei et al., 2003). Furthermore, in several countries, 2–11% of commercial pig livers or pork liver at slaughterhouse have been shown to be contaminated with HEV (Feagins et al., 2007, 2008; Rose et al., 2011; Wenzel et al., 2011). In the absence of a practical cell culture system, that can facilitate the propagation of HEV in vitro, many aspects of
the HEV life cycle are still not well understood. Models of HEV culture in vitro have been attempted in primary hepatocytes from non-human primates (Tam et al., 1996, 1997) and in various continuous cell lines, such as human hepatoma cell lines (PLC/PRF/5, HepG2 and Huh-7 cells), human colon carcinoma cells (Caco-2) and human lung cancer cells (A549) (Emerson et al., 2004, 2010; Tanaka et al., 2007). However, none of these culture systems can provide high-titres of infectious HEV in the culture supernatant. More recently, improved models were developed in PLC/PRF/5 and A549 (Tanaka et al., 2007), but these cell culture systems cannot mimic the events which occur during a natural HEV infection in vivo since the PLC/PRF/5 cell line contains several integrated HBV genes, which may affect HEV replication, and A549 is a non-hepatic cell line.

In the present study, two new cell lines were selected for their high similarity with human and porcine primary hepatocytes. First, the human hepatoma-derived cell line, HepaRG, which is able to differentiate into both biliary and hepatocyte-like cells was selected and secondly the PICM-19 cell line, derived from the primary culture of pig embryonic stem cells, which is also able to form either bile ductules or hepatocyte monolayers in vitro. Both HepaRG and PICM-19 cell cultures were shown to have inducible cytochrome P450 (CYP450) activity, and exhibit similar gene expression to primary human hepatocytes (PHHs) or primary porcine hepatocytes (PPHs) (Cerec et al., 2007; Hart et al., 2010; Talbot et al., 2010; Willard et al., 2010). HEV replication was assessed in these two culture systems and the results presented here clearly show the complete replication of HEV with the release of encapsidated RNA into the cell culture medium. HEV infection was inhibited by anti-HEV antibodies, suggesting specific virus–cell interactions. Thus, HepaRG and PICM-19 cells provide attractive alternatives to PHHs or PPHs and represent promising cellular models to study HEV virus–host interactions and species barrier crossing.

RESULTS

HepaRG and PICM-19 stay in a hepatocyte differentiation state during the infection assay

Since the extracellular matrix modulates various cellular processes including differentiation and polarization (Castell & Gómez-Lechón, 2009), HepaRG cells were cultured and embedded in 3D Matrigel. The presence of putative polarized hepatocyte-like cells forming bile canaliculi, which are constituted by lateral tight junctions between adjacent hepatocytes, was observed at 21 days post-plating and became optimum at 28 days post-plating (Fig. 1a). Some biliary-like cells were also observed. PICM-19 cells spontaneously differentiated into hepatocyte-like cells with bile canaliculi on an STO feeder cell (a mouse embryonic fibroblast line) layer by 10 days post-plating (Fig. 1b). Then, the differentiated and polarized cells were infected with HEV at an m.o.i. of 0.5 and infection was monitored for 35 days and the maintenance of the state of cell differentiation as functional hepatocytes was evaluated during infection. The kinetics of expression of hepatocyte-specific genes, albumin, transferrin and CYP3A4, were analysed. As shown in Fig. 1(c), all genes were expressed throughout the infection in both cell systems. However, the gene expression of transferrin and albumin were slightly reduced after 15 days post-infection (p.i.) in HepaRG cells, suggesting a decreased differentiation status in the hepatocyte-like cells. The CYP3A4 gene expression in HepaRG and its counterpart in porcine cells were detected from day 1 to 35 days p.i.

Detection of HEV RNA in the supernatant of infected cells

After HEV inoculation, the presence of viral RNA was followed in the cell culture supernatants for 35 days p.i. High levels of viral RNA were detected on day 1 p.i., probably reflecting viral overload from the inoculum. Then, the number of genome equivalents (GE) decreased at 4 days p.i., increased by 8 days p.i. and reached a maximum at 32 days p.i. in the culture supernatant of both HepaRG and PICM-19 cells with $1 \times 10^3$ and $450 \times 10^2$ GE ml$^{-1}$, respectively (Fig. 2).

To confirm that the detected RNAs were corresponding to encapsidated RNA, and possibly infectious particles, an RNase treatment was performed to remove free RNA or RNA from degraded particles. In the inoculum sample (faecal suspension), which contains intact infectious viral particles, HEV RNA quantification was not affected by RNase treatment (Fig. 3a). In contrast, RNase treatment resulted in the absence of detection of HEV RNA using free in vitro-transcribed RNA (Fig. 3a).

When the culture supernatants of HepaRG (Fig. 3b) and PICM-19 (Fig. 3c) -infected cells were treated or not with 0.1 U RNase, no significant differences were observed between the RNA quantified in RNase-treated and untreated supernatants, indicating that all viral RNA quantified in the supernatants of both cell culture systems, originated from intact viral particles.

Intracellular replication of HEV in HepaRG and PICM-19 cells

To evaluate HEV replication, the kinetics of intracellular levels of HEV RNA was measured. A gradual increase in viral RNA was observed in HepaRG cells (from $4.5 \times 10^2$ GE ml$^{-1}$ at 1 day p.i. to $4.6 \times 10^4$ GE ml$^{-1}$ at 21 days p.i.) (Fig. 4a). In PICM-19 cells, the number of GE reached a maximum of $6.8 \times 10^4$ as soon as 8 days p.i. and then remained approximately constant until 35 days p.i. (Fig. 4a).

Presence of HEV RNA negative-strand, a proof of HEV genome replication, was also evaluated in both culture models. Negative-sense HEV RNA was detected 8 days p.i.
in infected HepaRG cells (Fig. 4b) and as soon as 1 day p.i. in infected PICM-19 cells. No negative-strand amplification was detected in mock-infected cells.

Moreover, viral protein expression in human and porcine hepatocyte cell lines was studied by immunofluorescence assay, 21 days p.i. Capsid antigens were detected in infected HepaRG and PICM-19 cells, but not in mock-infected cells (Fig. 5). Staining was observed in the cell cytoplasm (Fig. 5).

**Effect of heat inactivation on HEV detection in supernatants of infected cells**

To determine whether the viral RNA detected in culture supernatants corresponds to an active multiplication of HEV in both systems, and not to an unspecified release of adsorbed viruses over time, HEV detection was estimated after heat treatments. Previously, a study had shown that HEV present in liver was resistant to heating at 56 °C, but was inactivated at 100 °C (Feagins et al., 2008). Thus, viral suspensions heated at 56 °C for 60 min or at 95 °C for 5 min were used to inoculate both cell culture models. HEV incubated at room temperature for 60 min was used as a control. The kinetics of HEV RNA detection was followed in the culture supernatants by real-time quantitative RT-PCR (qRT-PCR). The levels of viral RNA at day 0 represents the viral concentration from each sample before heat treatments, and the levels of viral RNA at 1 day p.i. correspond to the viral overload from each inoculum (Fig. 6a, b). When the inocula were heated to 56 °C or 95 °C, viral RNA was not detected in the supernatant of the two culture systems at 4 days p.i., indicating that HEV was inactivated at 56 °C for 60 min and at 95 °C for 5 min (Fig. 6a, b). In contrast, when the virus did not undergo heat treatment, viral RNA was detected throughout the infection period in the culture supernatant of HepaRG, with a growing phase starting at 8 days p.i. (Fig. 6a). Similarly, in the culture supernatant of PICM-19, viral RNA was detected at 8 days p.i. and increased with time (Fig. 6b).
Inhibition of HEV infection using anti-HEV antibodies

To confirm the specificity of HEV infection of these two cell culture systems, swine serum samples containing or not anti-HEV antibodies were used to neutralize HEV infection. HEV inoculum was pre-incubated with serum samples positive or negative for anti-HEV antibodies and used to infect HepaRG or PICM-19 cell cultures. HEV RNA disappeared at 12 or 8 days p.i. in supernatant culture of HepaRG (Fig. 7a) and PICM-19 (Fig. 7b), respectively. However, with the control pig serum (anti-HEV-negative), HEV RNA continued to be detectable up to the end of the infection period in both culture systems (Fig. 7c). Moreover, negative-sense HEV RNA was detected in infected cells only when the inoculum was pre-incubated with the negative control serum (Fig. 7c). Taken together, these results confirm that the capacity of HEV to infect HepaRG and PICM-19 cells involve specific interactions.

Fig. 2. Detection of HEV RNA in culture supernatants. HepaRG or PICM-19 cells were inoculated with HEV at an m.o.i. of 0.5 or mock infected and cultured for the indicated number of days. HEV RNA present in supernatants was quantified by real-time RT-PCR. Results are expressed as the mean of three independent experiments ± SEM.

Fig. 3. HEV RNA quantified in the supernatant after RNase treatment. (a) Inoculum (faecal suspension containing HEV) and in vitro-transcribed RNA (10⁶ copies) were treated with different concentrations of RNase A (0.01 and 0.1 U). HEV RNA was quantified by real-time RT-PCR (GE ml⁻¹). HepaRG cells (b) or PICM-19 cells (c) were inoculated with HEV at an m.o.i. of 0.5 or mock infected and cultured for the indicated number of days. Cell culture supernatants were collected and treated or not treated with 0.1 U of RNase A, and HEV RNA was quantified by real-time RT-PCR. Results are expressed as the mean of three independent experiments ± SEM.
DISCUSSION

Hepatitis E can be a zoonotic disease in industrialized countries and swine population is a major HEV reservoir for human contaminations. As with hepatitis viruses, HEV does not grow efficiently in vitro, and few data are available on the molecular mechanisms underlying HEV virulence and interspecies transmission. The present study demonstrates that two new models, based on the human hepatoma-derived cell line, HepaRG, and the porcine embryonic stem cell-derived cell line, PICM-19, can support a complete replication of HEV. The intracellular replication of HEV was shown by detecting increasing amounts of total HEV RNA

![Fig. 4. Intracellular replication of HEV in HepaRG and PICM-19 infected cells. Detection of HEV RNA (GE ml⁻¹) in HepaRG and PICM-19 cells at 1, 8, 14, 21, 28 and 35 days p.i. (a) Total HEV RNA was quantified by real-time RT-PCR. Results are expressed as the mean of three independent experiments ± SEM. (b) The negative-strand HEV RNA in HepaRG cells (upper panel) or PICM-19 cells (lower panel) was detected by amplification of a 286 bp ORF1 partial sequence after cDNA of the negative strand was synthesized.](http://vir.sgmjournals.org)

![Fig. 5. Immunofluorescence staining of HEV structural protein. HepaRG (two upper panels) and PICM-19 (two lower panels) cells infected or not with HEV and stained 21 days p.i. with a rabbit polyclonal anti-HEV capsid (middle panel). Nuclei were counterstained with DAPI (left panel). In the merged images (right panel), nuclei were visualized in blue and viral proteins in green. Bar, 10 μm.](http://vir.sgmjournals.org)
and detection of negative-strand RNA. Moreover, HEV capsid protein was detected in infected cells by immunofluorescence. The release of intact viral particles was also shown in culture supernatants by detecting HEV RNA after RNase treatment. Absence of HEV release was observed both after thermal inactivation or neutralization with anti-HEV antibodies, confirming the specificity of the two infection models.

In comparison to the other models recently improved in the human cell lines A549 and PLC/PREF5 (Takahashi et al., 2010; Tanaka et al., 2007), the quantity of viral RNA released in the supernatants of infected HepaRG and PICM-19 cell cultures was lower (10^3 versus 10^7). However, the m.o.i. used in the other models was not clear and high initial titres were required.

Using these two new models, HEV suspensions heated at 56 °C for 60 min, were shown to be non-infectious. This result is different from previous studies, where HEV genotype 1 or HEV genotype 3 present in pork liver or stool sample were still infectious after 56 °C treatment (Emerson et al., 2005; Feagins et al., 2008; Tanaka et al., 2007). However, these previous studies were performed with either different sample origins (liver versus aqueous solution), different incubation times (30 min versus 60 min) or different genotypes (1 versus 3) that may affect HEV resistance to thermal treatment.

The infectivity of the new progeny virions released in cell culture supernatants was assessed, but the low m.o.i. used failed to produce any infection.

Even so, in comparison to other models developed earlier (for review see Okamoto, 2011), Matrigel-embedded HepaRG and PICM-19 cell cultures possess several advantages. Indeed, the HepaRG and PICM-19 cells maintained a hepatocyte-like morphology and liver-specific gene expression throughout the infection, which is important for dissecting the mechanisms of virus-target–cell interactions. Moreover, HepaRG and PICM-19 cells displayed in vitro ultrastructure, enzymic and transport characteristics similar to those of primary hepatocytes (Talbot et al., 1996a; Le Vee et al., 2006). As previously observed in vitro and in vivo (Emerson et al., 2004; Purcell & Emerson, 2008), HEV infection led to non-cytopathic effects.

By mimicking the events that occur during natural infection of HEV in vivo, these two models may enable investigations on the cell signalling pathways modulated by HEV, the HEV intracellular traffic or the secretion pathways taken by virions, none of which are fully understood.

The HepaRG and PICM-19 cell lines will allow further investigations on the zoonotic aspects of HEV infection and possible species-specific host factors involved in HEV multiplication. Indeed, it has been shown that a genotype 3 strain isolated from a chronically infected patient (Kernow C1) and the US2 strain isolated from an infected patient and after inoculation to a rhesus macaque, could infect swine kidney cells (LLC-PKS) more efficiently than human cells (HepG2/C3A). Thus, it suggests that species-specific factors may affect HEV infections (Shukla et al., 2011). In the present study the swine HEV of genotype 3f used was shown to have infected human and porcine hepatocyte-like cells with similar efficiency. This is in accordance with a recent study showing the absence of a species barrier and a clear adaptation of genotype 3f HEV to both humans and pigs using in vivo transmission from human to swine (Bouquet et al., 2012).

In conclusion, HepaRG and PICM-19 cell cultures provide useful tools to improve the understanding of the HEV replication cycle in the context of differentiated human or porcine hepatocyte-like cells. These culture systems may enable the identification of the host proteins involved in HEV entry and exit processes, and in HEV replication.
They may also be useful to evaluate the level of virulence or the zoonotic potential of different HEV strains.

**METHODS**

**Virus.** Swine faecal samples containing HEV of genotype 3, subtype 3f (GenBank accession no. JN906976) were collected from experimentally infected pigs (Bouquet et al., 2012). Faecal suspensions were prepared [1 or 2 g in 10% (v/v) phosphate buffer] and centrifuged at 4000 g at 4 °C for 20 min. The resulting clear supernatant was purified by passage through microfilters with a pore size of 0.45 and 0.22 mm (Millex-GV; Millipore SAS). The quantity of HEV was estimated to be 10^8 copies of HEV genome equivalents (GE g^-1) using real-time quantitative RT-PCR (qRT-PCR) as described below. Aliquots of suspension were stored at -80 °C until use.

**Cell cultures.** HepaRG cells (BIOPREDIC International) were maintained in William's E medium (Fisher Scientific) supplemented with 10% (v/v) FBS, 100 U penicillin ml^-1, 100 μg streptomycin ml^-1, 5 μg insulin ml^-1 (Sigma-Aldrich) and 5 x 10^-5 M hydrocortisone hemisuccinate (VWR International).

The derivation of the PICM-19 cell line was previously described and its continuous culture was performed as previously described (Talbot et al., 1993, 1994). PICM-19H cells were continuously cultured on irradiated (8 krad) STO mouse fibroblast (CRL 1503; American type Culture Collection) feeder cell layers. The growth and differentiation medium was a 50:50 mixture of low glucose Dulbecco's modified Eagle's medium (DMEM; <supplier>) and Medium 199 (Fisher Scientific) with 10% FBS supplementation [10% (v/v) DMEM/199; Talbot et al., 1996b], with which cells were fed every 2 days after passage. Stock PICM-19H cells were routinely divided every 2 weeks for maintenance in continuous culture at 37 °C and 5% CO2.

For 3D Matrigel-embedded cultures of cell lines, 1 ml of complete medium containing 2 x 10^5 cells was added to 200 μl of the extracellular matrix derived from Engelbreth-Holm-Swarm mouse tumour (Matrigel; Sigma-Aldrich), gently mixed and coated into a six-well plate. After incubation at 37 °C for 20 min, 10^5 cells in 1 ml of medium culture was added to each well. Cells were grown at 37 °C under 5% CO2 atmosphere for 3 weeks, changing medium every 2 days.

**Virus inoculation.** HepaRG cells in six-well plates and PICM-19 in T12.5 flasks were infected with HEV at an m.o.i. of 0.5 and incubated overnight at 37 °C in a humidified 5% CO2 atmosphere. The viral suspension was then removed and cells were washed three times with 2 ml PBS before adding 2 ml of maintenance medium. The maintenance medium of HepaRG cells consisted of William's E medium supplemented with 2% FBS, 100 U penicillin ml^-1, 100 μg streptomycin ml^-1, 5 μg insulin ml^-1 and 5 x 10^-5 M hydrocortisone hemisuccinate, whereas for PICM-19 cells it was their growth and differentiation medium. Every 2 days, one-half (1 ml) of the culture medium was collected and replaced with fresh maintenance medium.

**HEV thermal inactivation.** Virus stock (400 μl) was incubated at 56 °C for 60 min or 95 °C for 5 min. As a control, 400 μl of virus stock was kept at room temperature for 60 min. The heat-treated and the control virus were inoculated on cells at an m.o.i. of 0.5 in a six-well plate for HepaRG cells or in T12.5 flasks for PICM-19 cells. The protocol after infection and maintenance of cultured cells was performed as described above.

**Neutralization assay.** A 0.2 ml aliquot of virus containing 10^6 copies of HEV GE was mixed with 200 μl of a pool of swine serum positive for anti-HEV antibodies or control swine serum (anti-HEV negative) diluted 1:10 in PBS and incubated at room temperature for 1 h and then inoculated on cells in a six-well plate for HepaRG cells or in T12.5 flasks for PICM-19 cells. After 24 h, the inoculum was removed and cells were rinsed three times with PBS before adding 2 ml of maintenance medium. Maintenance of cell culture after infection was performed as described above.
RNase treatment and nucleic acid extraction from supernatant.
One unit of RNase A (Qiagen) diluted 1:10 or 1:100 with nuclease-free water was added to 200 μl of each sample (HEV RNA standard or faecal suspension) and incubated at 37 °C for 30 min. Thereafter, RNase A activity was inhibited by the addition of 18 U of RNase inhibitor (Qiagen) and incubation at room temperature for 30 min. The RNAs were then extracted using the QIAamp Viral RNA Mini kit (Qiagen) as described below, except β-mercaptoethanol diluted 1:200 was added in AVL buffer (QIAamp Viral RNA Mini kit; Qiagen). Finally, 8 U RNase inhibitor were added to the extracted RNA.

Nucleic acid extraction from infected cells. Totals RNAs from pelleted cells were extracted using the RNasey Minikit (Qiagen). Viral RNAs were extracted from culture supernatants using the QIAamp Viral RNA Mini kit according to the manufacturer’s protocol except that extraction was performed on 200 μl of culture supernatant or faecal samples (10 % PBS, pH 7.2) with 800 μl of AVL buffer with carrier. RNAs were eluted twice with 40 μl sterile water after 1 min of incubation at room temperature. Aliquots of RNAs were stored at −80 °C until use.

RT and PCR controls. Precautions were taken to prevent false-positive and false-negative results in RT-PCR amplification. In addition to spatial separation of workspaces at crucial experimental points (e.g. RNA extraction, PCR mix preparation), each experiment included several control samples: positives samples for RNA extraction; negative and positives controls of RT, first PCR and nested PCR in each run.

Detection of HEV negative-strand RNA. Complementary DNA of the negative strand was synthesized using a strand-specific primer: conS1 (Forward, 5’-CTGGCATYACTACTGCGATTGACG-3’). Reverse transcription was performed at 42 °C for 60 min with 5 μl of total RNA (template), 2.5 μl of reverse primer of ORF1, 60 U PrimeScript Reverse Trans Takara in RT buffer (Ozyme), 1 mM deoxyribonucleotide triphosphates (dNTP) mix (Ozyme), and 12 U RNase inhibitor (Life Technologies). The reverse transcriptase activity of PrimeScript Reverse Trans Takara was then heat-inactivated at 72 °C for 10 min. Then a nested PCR, amplifying a region within the logarithm of the input copy numbers of standard RNA. Standard quantification curves, the CP values were plotted proportionally to the crossing points (CP) were calculated. For generation of standard quantification curves, the CP values were plotted proportionally to the logarithm of the input copy numbers of standard RNA. Standard RNA was obtained after in vitro transcription of a plasmid pCDNA 3.1 ORF2-3 HEV, as described by Barnaud et al. (2012).

Detection of mRNA of albumin, transferrin, actin and CYP450. cDNA synthesis was performed from 400 ng total RNA at 42 °C for 60 min with 2.5 μl hexamer, 60 U PrimeScript Reverse Trans Takara in RT buffer, 1 mM dNTP mix and 12 U RNase inhibitor. For each liver-specific transcript (albumin, transferrin, actin and CYP450 enzyme CYP3A) 5 μl cDNA was amplified by PCR using specific primers (Cerec et al., 2007; Thörn et al., 2011; human CYP3A4: forward primer 5’-CTTCATCAATGAGACTCAATAT-3’, reverse primer 5’-TCCGCTGATACGCTGCGGAA-3’; porcine CYP3A: forward primer 5’-CCATGAAATCTGTACGCTGGCAG-3’, reverse primer 5’-GGCTGACTGACGCTGGG-3’; human albumin: forward primer 5’-ATACAAAACAGTGCCAGACGAGCAC-3’, reverse primer 5’-CCGCAAGGACCAGGACACTCAG-3’; β-actin: forward primer 5’-CCGCAAGGACCAGGACACTCAG-3’, reverse primer 5’-GGCTGACTGACGCTGGG-3’). Reactions were performed in a 50 μl reaction mixture containing 5 μl of the resulting first-round PCR product (template), 1 U Platinum DNA polymerase enzyme, in 1× Platinum buffer, 1.5 μl MgCl2 mix (50 mM) and 0.2 μM of each forward and reverse primers. The PCR parameters for the second PCR round were an initial denaturation step at 94 °C for 1 min, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C and an extension at 72 °C for 30 s, and followed by a final extension at 72 °C for 10 min. The expected final product of the nested RT-PCR is 287 bp. Amplified products were visualized after agarose (1.5 %) gel electrophoresis and ethidium bromide staining. Negative controls of RT, first PCR and nested PCR were included in each run.

Immunofluorescence staining. HepaRG and PICM-19 cells were grown on glass coverslips (18 mm diameter) placed individually in 12-well tissue culture plates and were infected or not with HEV at m.o.i. of 10. The infection assay was performed as described above. At 21 days p.i., cells were washed three times with PBS, fixed with 4 % paraformaldehyde for 5 min at room temperature, and again washed with PBS. The permeabilization and blocking steps were carried out with 5 % BSA (Sigma) in PBST (0.01 % Triton X-100 in 1× PBS) for 10 min at room temperature. After blocking, cells were incubated for 90 min at room temperature with a rabbit polyclonal anti-HEV (1:100 dilution in 5 % BSA-PBST). This antibody was kindly provided by Dr Nathalie Ruvoen and Monica Lora from ONIRIS, Nantes. Following three washes with 5 % BSA-PBST, cells were incubated with goat anti-rabbit immunoglobulin G conjugated with Alexa-488 (1:200; Life Technologies) for 1 h at room temperature. Cells were then washed three times with PBST and slides were covered with Vectashield mounting solution containing DAPI (Vector Laboratories). Samples were examined using a Zeiss inverted Axio Observer microscope using ×63 oil immersion objective.

TaqMan real-time RT-PCR assay. HEV RNA quantification was adapted from the method described by Jothikumar et al. (2006) and performed as described in Barnaud et al. (2012). Briefly, TaqMan RT-PCR was performed using the QuantiTec Probe RT-PCR kit (Qiagen) according to the manufacturer’s instructions with 2 μl RNA (template). Reverse primer (5’-AGGGGTTGGTTGATGAA-3’) was used at a final concentration of 0.25 μM, forward primer (5’-GGTGTGTTTCTGGGTTGAC-3’) at 0.1 μM and probe (FAM-TGATTTCTGCAGCTTGG-MGB) at a final concentration of 5 μM. A LightCycler apparatus (Roche Molecular Biochemicals) was used for sample analysis. Reverse transcription was carried out at 50 °C for 20 min, followed by denaturation at 95 °C for 15 min. cDNA was amplified immediately with 45 cycles at 95 °C for 10 s and 58 °C for 45 s. The final extension was followed by cooling at 40 °C for 30 s. Real-time RT-PCR data were collected after the reaction and the crossing points (CP) were calculated. For generation of standard quantification curves, the CP values were plotted proportionally to the logarithm of the input copy numbers of standard RNA. Standard RNA was obtained after in vitro transcription of a plasmid pCDNA 3.1 ORF2-3 HEV, as described by Barnaud et al. (2012).
denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and an extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. Amplified products were visualized after agarose (1.5 %) gel electrophoresis and ethidium bromide staining.

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