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Viral Hepatitis

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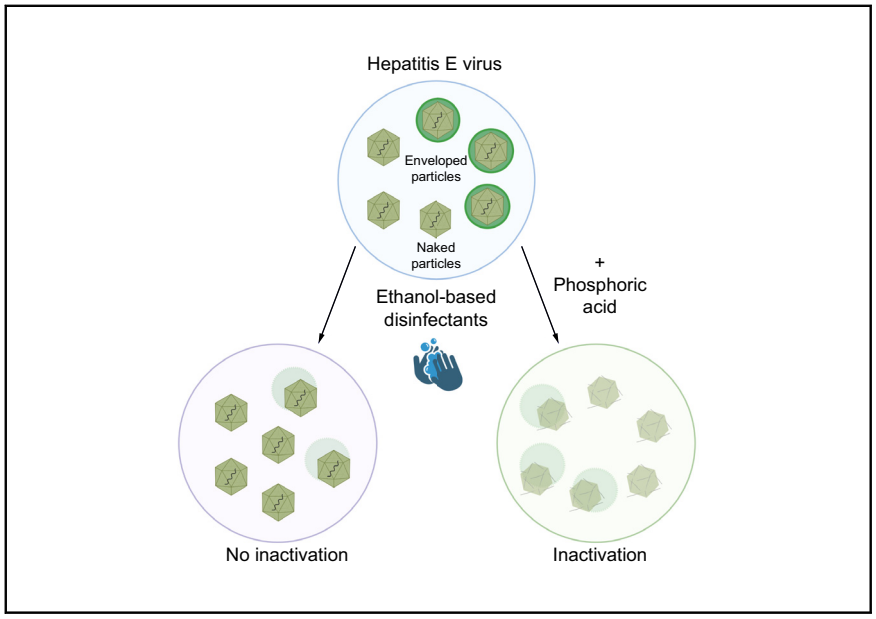
Graphical abstract



Highlights

- HEV is highly resistant to inactivation by alcohols and commercially available alcohol-based disinfectants.
- Phosphoric acid as a supplement in alcohol-based disinfectants plays an important role in virucidal activity against HEV.
- Ethanol disrupts the quasi-envelope of HEV while leaving the infectious naked virions intact.

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Lay summary

Hepatitis E virus (HEV) showed a high level of resistance to alcohols and alcohol-based hand disinfectants. The addition of phosphoric acid to alcohol was essential for virucidal activity against HEV. This information should be used to guide improved hygiene measures for the prevention of HEV transmission.

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Hepatitis E virus is highly resistant to alcohol-based disinfectants

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Background & Aims: Hepatitis E virus (HEV) is the most common cause of acute viral hepatitis worldwide and is mainly transmitted via the fecal-oral route or through consumption of contaminated food products. Due to the lack of efficient cell culture systems for the propagation of HEV, limited data regarding its sensitivity to chemical disinfectants are available. Consequently, preventive and evidence-based hygienic guidelines on HEV disinfection are lacking.

Methods: We used a robust HEV genotype 3 cell culture model which enables quantification of viral infection of quasi-enveloped and naked HEV particles. For HEV genotype 1 infections, we used the primary isolate Sar55 in a fecal suspension. Standardized quantitative suspension tests using end point dilution and large-volume plating were performed for the determination of virucidal activity of alcohols (1-propanol, 2-propanol, ethanol), WHO disinfectant formulations and 5 different commercial hand disinfectants against HEV. Iodixanol gradients were conducted to elucidate the influence of ethanol on quasi-enveloped viral particles.

Results: Naked and quasi-enveloped HEV was resistant to alcohols as well as alcohol-based formulations recommended by the WHO. Of the tested commercial hand disinfectants only 1 product displayed virucidal activity against HEV. This activity could be linked to phosphoric acid as an essential ingredient. Finally, we observed that ethanol and possibly non-active alcohol-based disinfectants disrupt the quasi-envelope structure of HEV particles, while leaving the highly transmissible and infectious naked virions intact.

Conclusions: Different alcohols and alcohol-based hand disinfectants were insufficient to eliminate HEV infectivity with the exception of 1 commercial ethanol-based product that included phosphoric acid. These findings have major implications for the development of measures to reduce viral transmission in clinical practice.

Lay summary: Hepatitis E virus (HEV) showed a high level of resistance to alcohols and alcohol-based hand disinfectants. The addition of phosphoric acid to alcohol was essential for virucidal activity against HEV. This information should be used to guide improved hygiene measures for the prevention of HEV transmission.

Introduction

Hepatitis E virus (HEV), genus *Orthohepevirus*, family *Hepeviridae*, is the most common cause of acute viral hepatitis worldwide. The mode of transmission varies greatly and is dependent on the circulating virus genotype in each region.[1] Overall, 4 major human-pathogenic genotypes (gt) exist: HEV-1 and HEV-2 circulate mainly in resource-limited countries and can lead to large outbreaks due to fecal-oral transmission. Humans are the only reservoir for these genotypes. One risk group for infection are pregnant women, in whom HEV is associated with relatively high mortality.[2] Conversely, HEV-3 and HEV-4 are zoonotic pathogens circulating mainly in developed regions, which have their main reservoir in domesticated pigs/wild boars and other animals.[1] In contrast to HEV-1 and HEV-2, they can cause sporadic infections transmitted by consumption of contaminated meat products or transfusion of HEV-positive blood products.

Keywords: hepatitis E virus (HEV); disinfection; alcohol; suspension assay; inactivation.
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Additionally, individuals with close contacts to infected animals such as hunters, veterinarians and farmers are at risk for acquiring HEV infection.[3]

Currently, the World Health Organization (WHO) as well as national health authorities recommend the implementation of water, sanitation and hygiene (WASH) interventions for HEV outbreaks.[4] In developed countries with autochthonous infections hygiene measures are usually improved by, for example, use of personalized toilets for patients, decreased or restricted person-to-person contact and improved hand hygiene. However, until now it is unclear which measures need to be taken for efficient disinfection. Commonly, reference viruses are used to reflect and extrapolate disinfection measures in national and international guidelines (*e.g.* German Guidelines DVV/RKI, European Norm EN 14476, U.S. Methods ASTM E1052-11).[5] HEV is usually circulating in the environment as naked virus and is assumed to have a higher stability and resistance to chemical disinfectants compared to enveloped viruses. However, in the blood, HEV circulates as a quasi-enveloped viral particle wrapped by a cellular membrane which might influence the activity of disinfectants.[6] Due to the lack of efficient cell culture systems for HEV, evidence-based hygiene guidelines and prevention measures are incomplete, and no data are available regarding HEV's sensitivity to chemical disinfectants. The quasi-enveloped nature of HEV complicates the implementation of guidelines as the enveloped and naked particles must be inactivated.

In this study, we utilized a recently developed robust HEV-3-based cell culture system for the evaluation of commonly used hand disinfectants and their principal components (*e.g.* alcohols) against the naked and quasi-enveloped form of HEV.[7] To verify translation of the results to a fecal-orally transmitted HEV genotype, pivotal experiments were conducted with a HEV-1-based primary isolate from stool samples (Sar-55).[8]

Materials and methods

Cell culture

The human liver cell line HepG2 were cultured in DMEM (Gibco, Thermo Fisher Scientific, Schwerte, Germany) supplemented with 10% FCS (GE Healthcare, Munich, Germany (Ref A15-151, Lot A15111-2028) or Capricorn Scientific, Ebsdorfergrund, Germany (Ref FBS11-A, Lot CP16-1377) or Gibco (Ref 10270-106, Lot 41Q1820K), 100 μg/ml of streptomycin and 100 IU/ml of penicillin (Gibco), 2 mM L-glutamine and 1% nonessential amino acids (Gibco) (DMEM complete). The HepG2/C3A subclone was cultured in Eagle's minimal essential medium (MEM with glutamine, Gibco), 10% ultra-low IgG FCS (Gibco, Ref 16250-078, Lot 1939770), 2 mM L-glutamine, 100 μg/ml gentamicin, 1 mM sodium pyruvate and 1% nonessential amino acids (Gibco) (MEM low IgG FCS medium).

HEV construct and *in vitro* transcription

A plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, HEV-3; GenBank accession no. JQ679013) with a point mutation in open-reading frame (ORF)-1 (G1634R) was used to generate HEV *in vitro* transcripts as previously described.[7,9,10] Capping of the constructs was performed using Ribo m7G Cap Analog (Promega, Madison, WI, USA).

Infectious cell culture-derived HEV-3 production assays

For transfection we used the electroporation technique in accordance with previous reports.[7,9] In brief, 9×10^6 HepG2 cells were resuspended in 400 μl cytomix containing 2 mM ATP and 5 mM glutathione, mixed with 5 or 10 μg of HEV RNA and subsequently electroporated. Cells were immediately transferred to 13.6 ml DMEM complete and the cell suspension was seeded in respective plates. After 24 hours the medium was changed to fresh medium. Viral particle production was determined at 7 days post transfection by harvesting the extracellular particles (which are quasi-enveloped virus particles) in the filtered (0.45 μm) supernatant. The intracellular virus (naked virus) was generated by resuspension of the cells in a 5 times lower volume of the respective supernatant and lysed by 3 repeated freeze and thaw cycles. After a high-speed centrifugation step, which separates the cell debris, the supernatant was harvested.

HEV-1 primary isolate

Primary HEV-1 strain Sar55 isolate in 10% fecal suspension from an infected macaque was kindly provided by Suzanne U. Emerson.[8]

HEV titration

To quantify the HEV infectivity titers, a serial dilution infection assay was performed using HepG2/C3A as target cells. The cells were seeded the day before with 2×10^4 cells/well in MEM low IgG FCS medium. Virus was serially diluted 3-fold and used for inoculation of the respective target cells. Seven days later viral titers were determined by indirect immunofluorescence staining of the viral capsid and counting the number of focus-forming units (FFUs) containing ORF-2 positive cells. FFUs were counted in 3 wells of highest dilutions where about 2-30 foci were detectable. The average of the counted numbers of the respective FFUs was calculated per milliliter. The lower limit of quantification (LLOQ) of respective titration assays was set to the lowest dilution with no cell death visible.

Indirect immunofluorescence assay

Cells were fixed at 7 days post transfection/infection, with 3% paraformaldehyde (PFA) in PBS, and stained as described previously.[7] Briefly, cells were stained for the ORF2-encoded capsid protein with an HEV capsid protein-specific rabbit hyperimmune serum and using a goat anti-rabbit antibody (AlexaFluor 488 or 568 for the trans-complementation assay, Life Technologies, Darmstadt, Germany) as secondary antibody. The rabbit hyperimmune serum was raised against a *Escherichia coli*-expressed and affinity chromatography-purified His-tagged HEV-3 capsid protein derivative, harboring amino acid residues 326–608, via subcutaneous immunizations at 4-week intervals.[11] This serum has been successfully used in immune electron microscopy and immunofluorescence assays.[12–14] DNA was labelled with DAPI (4′,6′-diamidino-2- phenylindole, Life Technologies).

Tested disinfectants

The principle components of the tested disinfectants were as follows: WHO formulation I: ethanol (80%), hydrogen peroxide (0.125%), glycerol (1.45%); WHO formulation II: 2-propanol (75%), hydrogen peroxide (0.125%), glycerol (1.45%); Hand disinfectant I: 78.2% (w/w) ethanol (96%), 0.1% (w/w) biphenyl-2-ol; Hand

disinfectant II: 45.0% (w/w) 2-propanol, 30.0% (w/w) 1-propanol, 0.2% (w/w) mecetroniumetilsulfate; Hand disinfectant III: 57.6% (w/w) ethanol (96%), 10% (w/w) 1-propanol; Hand disinfectant IV: 70% (vol/vol) 2-propanol; Hand disinfectant V: 95.0% (w/w) ethanol (99%). Tested acids were titrated in distilled aqua to pH 2.5 using either 37.5% HCl, 80% H_3PO_4, or 100% HCH_3COOH and respective volumes were used analogously to acidify 100% ethanol or PBS. All solutions were sterile filtered before use.

Heat inactivation

For heat inactivation, virus stocks or primary stool suspensions were prepared as described and subsequently incubated at 85 °C for 20 minutes before suspension or large-volume plating (LVP) assays.

Quantitative suspension assays

End point dilution method: For *in vitro* inactivation of HEV, experiments were carried out by mixing 1 part of test virus suspension with 1 part of organic load (BSA 0.3%, clean conditions) and 8 parts of the different kinds of alcohol, WHO formulations, commercially available hand disinfectants or water as control. After a short exposure time of 30 seconds, test mixtures were immediately diluted in DMEM (to stop the activity of the biocides) and then used for inoculation of target cells.

LVP represents an accepted method to increase the overall measuring window as well as sensitivity for tests when evaluating activity of disinfectants against different pathogens.[15] For HEV-3 inactivation, experiments were carried out by mixing 1 part of test virus suspension with 1 part of organic load (BSA 0.3%) and 8 parts of the different hand disinfectants. For HEV-1 inactivation, experiments were carried out by mixing 15 µl HEV primary isolate HEV-1 strain Sar55 stool suspension with 60 µl 100% ethanol or PBS. After 30 seconds, these mixtures were immediately diluted 1:100 (ethanol, disinfectant III), 1:200 (disinfectant II), 1:1,000 (disinfectant IV, HEV-1) or 1:2,000 (disinfectant II) in medium to stop action of disinfectants and transferred onto target cells in up to 1.5-2.5×96 well plates (3 plates for HEV-1) with 100 µl (200 µl for HEV-1) volume per well. Thereby, the dilutions ensured no cytotoxic effects on the target cells.

Seven days later, FFUs were determined by immunofluorescence staining as described above. In case residual infection could be detected, the following equation was used to determined infectious virus particles:[15]

$$c = \frac{D}{Vw}\left(-ln\frac{n-np}{n}\right)$$

where: c = concentration of the infectious virus particles; *D = dilution; Vw = volume per well; n = number of inoculated wells*; *np = number of virus-positive wells*

In case no residual infection was detected, the Poisson formula was applied to determine the limit of detection at a 95% probability:[15]

$$c = lnp/-V$$

where: *p = probability of not detecting a virus (here, the probability of not finding a virus must not be higher than 5% (p =0.05))*; *c = concentration of infectious virus particles; V = test volume*

Iodixanol density gradient fractionation

Density gradient centrifugation was performed as recently described.[16] In brief, samples were divided into 2×1 ml aliquots. Fractionation was performed by overnight centrifugation through an iodixanol step gradient (0–40%) at 154,000×g in a TH-641 swing-out rotor at 4 °C using a Sorvall Ultra WX80 centrifuge. Thereafter, 10×1 ml fractions were collected and levels of viral RNA and viral infectivity of each fraction were determined. Buoyant densities were analysed using a refractometer.

Quantitative real-time PCR

For detection of cell-free viral RNA in gradient fractions, we used the QIAamp Viral RNA Mini Kit (QIAGEN Inc, Valencia, CA, USA) for extraction, followed by detection of HEV RNA by the FTD Hepatitis E RNA kit (FTD, Fast-track Diagnostics Sàrl, Esch-sur-Alzette, Luxembourg) according to manufacturer's instructions on a Light-Cycler 480 (Roche, Mannheim, Germany) as previously described.[17]

Results

Susceptibility of HEV to 1-propanol, 2-propanol and ethanol

The tested alcohols 1-propanol, 2-propanol and ethanol, which either differ in total number of carbons (ethanol *vs.* propanol) or positioning of the hydroxy group (1-propanol *vs.* 2-propanol), are most widely used for commercially available hand disinfectants. In order to assess whether these components have virucidal activity towards naked and quasi-enveloped HEV, a standardized suspension assay (European Norm EN 14476) was used to evaluate liquid disinfectants. Briefly, the tested alcohol (8 parts) was mixed with a cell culture-derived virus stock (1 part) and BSA (1 part) as soil load (clean conditions). This mixture was incubated for 30 seconds and afterwards the residual virus was determined by titration on the target cells. As depicted in Fig. 1A, HEV infectivity was not affected by concentrations of 1-propanol ranging from 20-80% (Fig. 1A, left panel). This was also the case for the quasi-enveloped particles (Fig. 1A, right panel). Similar results of a resistant phenotype of HEV infectivity were obtained for 2-propanol (Fig. 1B) and ethanol (Fig. 1C). At higher concentrations of ethanol, a 5-fold reduction of HEV infectivity could be observed. However, infectivity of titers of 10^5 FFUs/ml could still be detected. As a control, effective inactivation of naked HEV particles could be demonstrated by heat treatment at 85 °C for 20 minutes (Fig. S1). To confirm this observed ethanol tolerance with another genotype, we next tested a HEV-1-based Sar55 primary isolate from a stool suspension derived from an infected rhesus macaque in a LVP suspension test. The use of LVP was necessary due to the expected lower titers not exceeding cell toxicity of the tested ethanol. Here, the test mixture was incubated for 30 seconds and then immediately diluted by the addition of medium. As depicted in Fig. 2, HEV-1 infectivity in the control was almost 10^3 FFUs/ml. Only a 5-fold reduction of HEV-1 infectivity was observed upon ethanol treatment, with detectable residual infectivity above the detection limit confirming the ethanol tolerance of HEV-1 (Fig. 2). Heat inactivation abolished HEV-1 infectivity completely. In summary, these results show that HEV demonstrated a high resistance to different alcohols.

Virucidal activity of WHO disinfectant formulations against HEV

In 2009, the WHO published 2 formulations for alcohol-based disinfections to be used in healthcare settings where

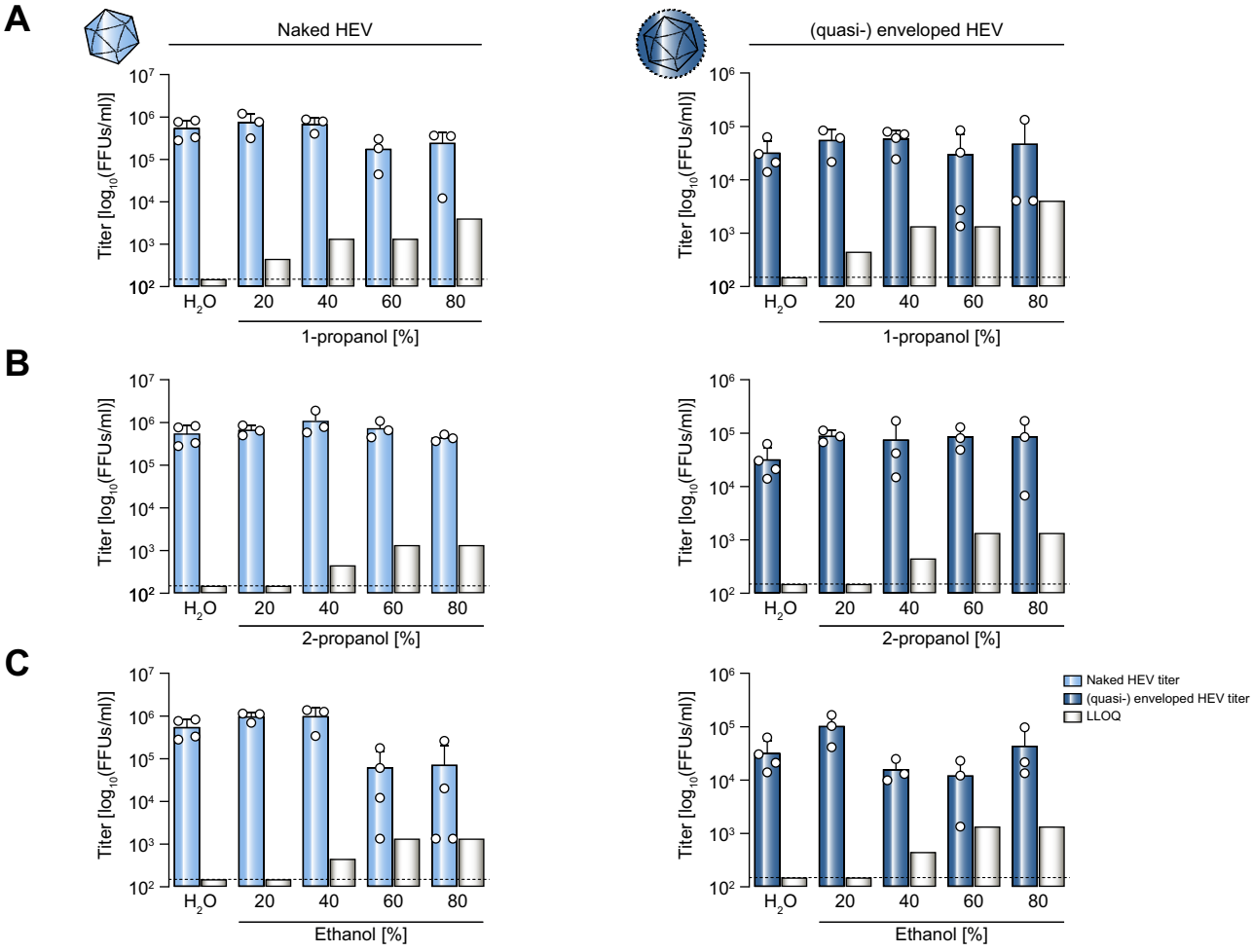

Fig. 1. Suspension test of alcohols against HEV-3. Cell culture-derived naked (left) and quasi-enveloped virus particles (right) were used in a standard suspension test to evaluate the virucidal activity of 1-propanol (A), 2-propanol (B) and ethanol (C) at different concentrations. Sterile water was used as negative control. Coloured bars represent infectious titer, white bars represent the LLOQ, the dotted line represents the detection limit of the assay (n = 3-4, means ± SD). FFUs, focus-forming units; LLOQ, lower limit of quantification.

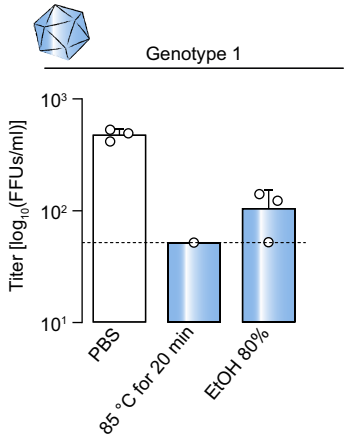

Fig. 2. Large-volume plating assay of ethanol against HEV-1. HEV primary isolate HEV-1 strain Sar55 stool suspension was used in a large-volume plating assay to evaluate the virucidal activity of ethanol. Sterile PBS served as negative control; heat inactivation for 20 minutes (min) at 85 °C was used for total inactivation. Coloured bars represent infectious titer, the dotted line represents the detection limit of the assay (n = 3, means ± SD). FFUs, focus-forming units; LLOQ, lower limit of quantification.

commercial hand rubs are too expensive or not available.[18] Formulation I is based on ethanol, whereas formulation II consists of 2-propanol. The original WHO formulations failed to meet the efficacy requirements of European Norm 1500 in previous tests.[19] However, Suchomel *et al.* suggested modified versions with increased concentrations of ethanol and 2-propanol, which we used in our study.[20] To determine the activity of these modified WHO formulations I and II against HEV, we incubated quasi-enveloped and naked viral particles for 30 seconds with the respective formulations (Fig. 3). In line with the results outlined above, no relevant reduction of HEV infectivity could be observed for the modified WHO formulations I and II in the suspension test (Fig. 3).

Virucidal activity of commercially available hand disinfectants against HEV

Next, we aimed to analyse the susceptibility of HEV to commercially available hand disinfectants. Therefore, we evaluated 5 different alcohol-based hand disinfectants (I-V). The main composition and the virucidal spectrum according to EN 14476 determined by reference viruses of the disinfectants are

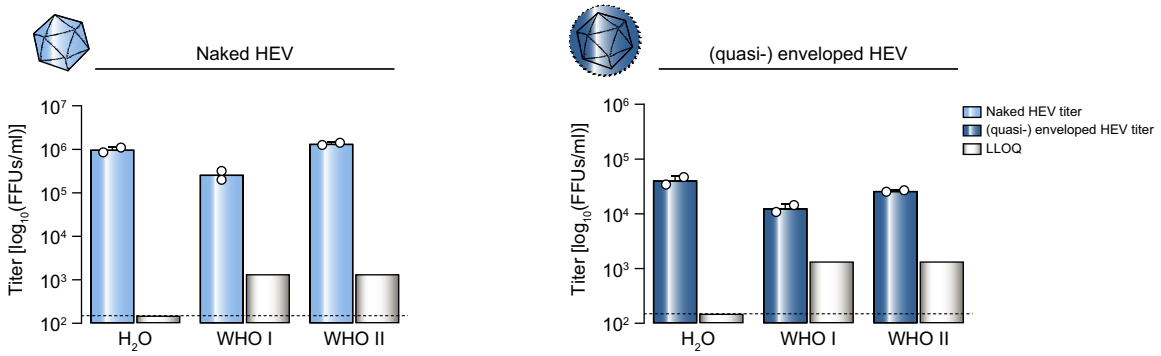

Fig. 3. Virucidal activity of modified WHO formulations against HEV. Cell culture-derived naked (left) and quasi-enveloped virus particles (right) was used in a standard suspension test (end point dilution) to evaluate the virucidal activity of modified WHO formulations. Sterile water was used as negative control (n = 2, means ± SD). FFUs, focus-forming units; LLOQ, lower limit of quantification; WHO, World Health Organization.

delineated in the materials and methods section. As depicted in Fig. 4A, a complete inactivation for the naked HEV particles to the limit of detection was only observed for product III (Fig. 4A, left panel). In all other cases HEV infectivity could still be detected (Fig. 4A). The evaluation of the quasi-enveloped virus particles revealed similar results, however, the measuring window was decreased because of the lower viral titers and high cytotoxicity of the disinfectants. To reduce cytotoxicity, we next performed LVP assays (Fig. 4B). Disinfectant V, which consists almost exclusively of ethanol, showed no effect on HEV infectivity in the standard suspension assay and was therefore not tested in the LVP. As a substitute, pure ethanol was used in LVP, which results in a final concentration of 80% in the assay solution (1-part BSA, 1-part virus and 8 parts ethanol). HEV infectivity comparable to the water control was detectable for ethanol and disinfectants I, II and IV using both types of viral particles (Fig. 4B). Only hand disinfectant III was able to completely disrupt viral infectivity of the naked and quasi-enveloped viruses, implicating only a selected susceptibility of HEV to commercially available alcohol-based hand disinfectants. Additionally, we evaluated 1-propanol, ethanol and 2-propanol as well as the 5 commercial hand disinfectants against hepatitis A virus – a liver-tropic virus that is transmitted via the fecal-oral route, known for a high environmental stability and quasi-enveloped nature like HEV. Naked hepatitis A virus was resistant to all 3 tested alcohols and all hand disinfectants (Fig. S2). In conclusion, of the 5 alcohol-based hand disinfectants tested, only a single product displayed virucidal activity against HEV.

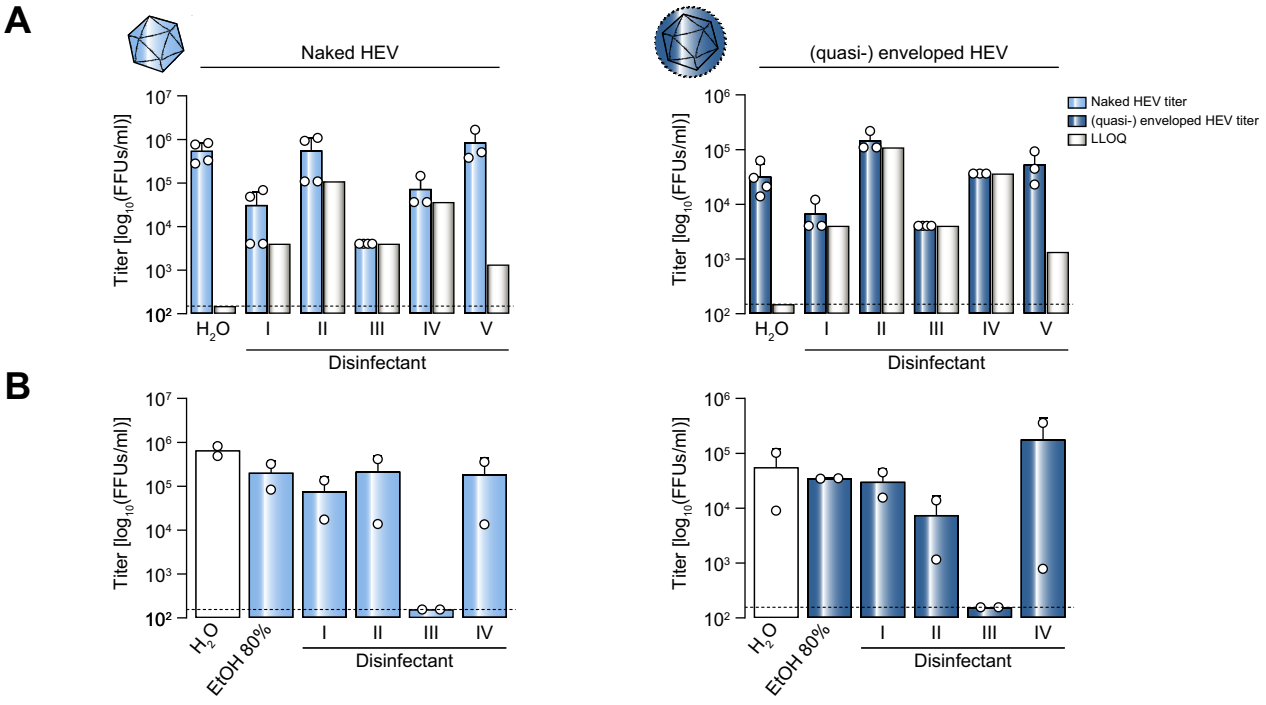

Fig. 4. Suspension test of commercially available hand disinfectants. Different commercial hand disinfectants were tested in the standard suspension test with end point dilution (A) or large-volume plating assay (B) using cell culture-derived naked (left) and quasi-enveloped virus particles (right). Sterile water was used as negative control. Coloured bars represent infectious titer, white bars the LLOQ and the dotted line the detection limit of the assay (n = 2-4, means ± SD). FFUs, focus-forming units; LLOQ, lower limit of quantification.

Different disinfectant formulations reveal phosphoric acid as an important ingredient for virucidal activity against HEV

Alongside alcohol, the different hand disinfectants include other compounds (*e.g.* acids, perfume etc.), which reconstitute less than 0.1% of the final product. These are usually not considered principal components of disinfectants, but may have an impact on their action against HEV. To elucidate the virucidal mode of action of product III, we next tested different formulations, lacking selected ingredients (Table 1). These formulations were evaluated in the quantitative suspension test using the LVP assay. The results showed that HEV infectivity could only be detected when phosphoric acid was depleted (Fig. 5A). To further evaluate the virucidal effect of phosphoric acid together with alcohols, we reversed the assay and instead of depleting phosphoric acid, phosphoric acid was combined with ethanol only. As depicted in Fig. 5B, the addition of phosphoric acid could improve the virucidal activity of 80% ethanol 10-fold. Next, we evaluated if other acids could improve the inactivation of HEV. The addition of acetic acid or hydrochloric acid to ethanol also resulted in an improved virucidal effect (Fig. S2). In summary, these results indicate that, in combination with alcohol, acids play an important role in virucidal activity against HEV.

Ethanol disrupts the quasi-envelope structure of HEV particles

While naked HEV demonstrated high resistance to ethanol and ethanol-based hand disinfectants, the quasi-enveloped particles even displayed a moderate increase in infectivity under certain conditions (Fig. 1A-C and Fig. 4A-B). To analyse if ethanol destroys the quasi-envelope structure and thereby generates naked intact HEV particles, we subjected ethanol-treated HEV particles to a density gradient analysis. As shown in Fig. 6, in the water-treated sample, viral RNA of quasi-enveloped particles peaked at a density of 1.15 g/ml (Fig. 6). After detergent NP40 (positive control) or ethanol treatment, these quasi-enveloped viruses were disrupted and shifted to the fractions of the naked form of HEV with densities above 1.2 g/ml (Fig. 6). Together, these results indicate that ethanol destroys the quasi-envelope of HEV particles and generates naked HEV particles.

Discussion

The recent establishment of an efficient HEV cell culture system allowed us to analyse the virucidal activity of alcohols and commercially available hand disinfectants against HEV.[7] We observed that HEV was not susceptible to the different components of commonly used disinfectants (1-propanol, 2-propanol and ethanol) with a proven activity against enveloped viruses and non-enveloped viruses following the European guideline. Three of the 5 commercially available ethanol-based products claim - according to testing with the European Norm – to be effective against enveloped and non-enveloped viruses. However, only one out of these tested hand disinfectants showed substantial activity against HEV. Interestingly, we could reveal that the addition of phosphoric acid was essential for the virucidal activity of the tested hand disinfectant. In a previous study, it was shown the basic formulation of the product, with ethanol 55% (w/w) and phosphoric acid 0.7% in combination with propan-1,2-diol and butan-1.3-diol, was responsible for its virus-inactivating activity against the stable poliovirus.[21] By utilizing a primary HEV-1 isolate (Sar55) we were able to demonstrate that this fecal-orally transmitted genotype was similarly resistant to the virucidal activity of ethanol.[8]

These findings need to be put into perspective according to the different lifecycle and risk for infection of the distinct genotypes of HEV. HEV-3 and HEV-4 are zoonotic infections in humans, usually transmitted via contaminated food products. However, individuals with close contact to possibly infected animals are at increased risk of acquiring HEV infection.[3] The use of gloves could reduce the risk of acquiring the infection in this setting.[22] Therefore, it is very likely that virucidal hand disinfectants including an activity against HEV also protect from infection in this risk group. On the flip side, HEV-1 and HEV-2 only circulate in humans and can cause large outbreaks in less-developed areas through waterborne transmission. Given that HEV-1 is highly resistant to ethanol inactivation, the development of hand disinfectants is highly relevant in addition to other measures in breaking the transmission chain. Therefore, we expect that the combination of different prevention measures (*e.g.* Swiss cheese model) like vaccine, food safety, sanitary hygiene and hand disinfectants will all contribute to the reduction of secondary infections independent of the HEV genotype. Proper hygiene measures to prevent virus spread are of utmost importance to reduce the overall burden of such outbreaks. The WHO has recommendations for disinfectant formulations which can be easily prepared and are available in more rural areas. Although we have recently observed strong virucidal activity against different enveloped[23] as well as non-enveloped stable viruses like poliovirus,[24,25] we show that these formulations are not able to disrupt the viral infectivity of HEV in the standard quantitative suspension assay – for both the naked and quasi-enveloped form of the virus particle. Therefore, knowing which components contribute to virucidal action against HEV is important and could be implemented into future recommendations.

Our cell culture model enabled the distinct evaluation of the naked and quasi-enveloped state of the virus particles. While in infected humans the naked virus is excreted via the faeces, the quasi-envelope virus circulates in the bloodstream. This type of particle in the blood seems to be protected from neutralizing antibody responses.[26] We observed no striking differences in terms of the virucidal activity of the tested disinfectants and principal components on the different forms of HEV particles. However, treatment with ethanol led to the destruction of the viral quasi-envelope structure. Intriguingly, the naked virus has a higher infectivity compared to the quasi-enveloped virus, possibly owing to a different mode of target cell entry.[27] Therefore, it is important to understand the mode of action and to test the effect of virus-inactivating components and solutions against

Table 1. Tested formulations of disinfectant III.

Formulations	Main ingredients	Changes to general formulation
VP-2069	Ethanol (50-60%); 1-propanol (9-11%)	Left out: butandiol-1,3; propylene glycol
VP-2070	Ethanol (50-60%)	Left out: 1-propanol
VP-2071	Ethanol (50-60%); 1-propanol (9-11%)	No changes
VP-2072	Ethanol (50-60%); 1-propanol (9-11%)	Left out: phosphoric acid

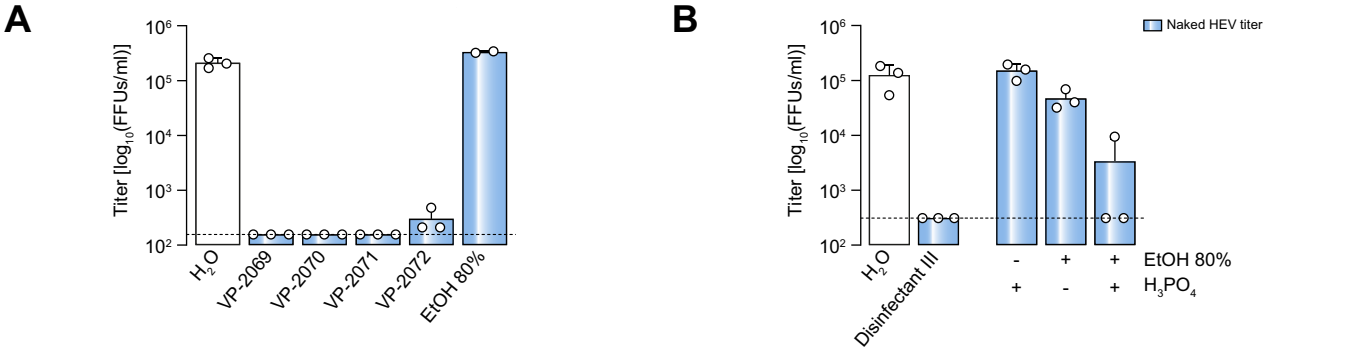

Fig. 5. Large-volume plating assay of different formulations of disinfectant III. Different formulations (VP) of disinfectant III were tested in a large-volume plating assay using cell culture-derived naked virus (A). Sterile water and ethanol (EtOH 80% final concentration) were used as controls. To evaluate the synergistic effect of ethanol and phosphoric acid in disinfectant III, both parts were tested separately or in combination (B). Coloured bars represent infectious titer, the dotted line represents the detection limit of the assay (n = 3, means ± SD).

HEV to exclude a possible enhancement of viral infectivity. For HEV-3 and HEV-4 it is still unclear whether patient to patient transmission is possible.[28] However, previous studies have clearly shown that viral particles which are secreted by infected individuals are infectious and can be transmitted to others via ingestion.[29] Due to the fecal-oral transmission of HEV-1 and HEV-2, which can cause waterborne outbreaks, the identification of active disinfectants to reduce HEV transmission is essential. Although we used a HEV-3-based model, our data can be translated to other genotypes as HEV circulates as 1 serotype with similar properties.[30] Additionally, until now, efficient cell culture models for other genotypes are lacking robustness in order to evaluate disinfectants.[31]

Overall, we provide evidence that HEV has strong stability against alcohols and alcohol-based hand disinfectants with the identification of 1 active product. These results can be used to improve hygiene measures for patients with an ongoing HEV infection. In particular, in cases of an HEV outbreak, all precautions should be made to reduce spread of the virus.

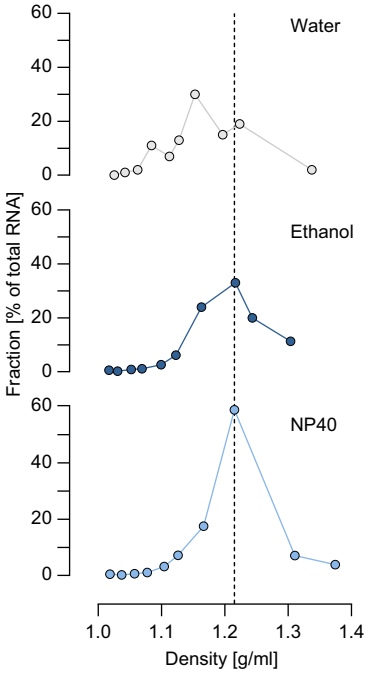

Fig. 6. Ethanol disrupts the quasi-envelope structure of HEV. Cell culture-derived quasi-enveloped virus particles were treated in the quantitative suspension test for 30 seconds with addition of ethanol (80% final concentration). After 30 seconds of incubation, the mixture was diluted with medium and subjected to an iodixanol gradient centrifugation. Ten fractions were harvested from the bottom and HEV RNA content was determined for each fraction. Percentages of total HEV RNA are depicted and plotted against density of the respective fraction measured by refractometry. Sterile water was used as a negative control. As a positive control, virus was incubated with the detergent NP40 to disrupt the quasi-envelope of HEV. One representative experiment out of 3 independent experiments is shown.

Abbreviations

FFUs, focus-forming units; HEV, hepatitis E virus; LLOQ, lower limit of quantification; LVP, large-volume plating; MEM, minimal essential medium; ORF, open-reading frame; WHO, World Health Organization.

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Conflict of interest

H.W.: Sponsored lectures/Consultant/Grants: Abbvie, Aligos, Altimmune, Biotest, BMS, BTG, Dicerna, Enanta, Gilead, Janssen, Merck/MSD, MYR GmbH, Roche, Vir Biotechnology. B.M.: Sponsored lectures/Consultant/Grants: AbbVie, Altona Diagnostics, Astellase, Fujirebio, Gilead, Medical Tribune, MSD, Roche Diagnostics, Roche. Stock or stock-options: BionTech.

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

Authors' contributions

PB and ES developed the concept, coordinated the study and wrote the manuscript. MF, JEW, VK, YS, DP, LH, BiB, BrB, PMN performed laboratory experiments and procedures. RGU provided essential reagents. PB, ES, BM, JS, FHHB, JS, YB, HW, DT

supervised the data collection and reviewed the drafts. All authors reviewed the final manuscript.

Data availability statement

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

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Supplementary data

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References

[1] Kamar N, Izopet J, Pavio N, Aggarwal R, Labrique A, Wedemeyer H, et al. Hepatitis E virus infection. Nat Rev Dis Primers 2017;3:17086.
[2] Bigna JJ, Modiyinji AF, Nansseu JR, Amougou MA, Nola M, Kenmoe S, et al. Burden of hepatitis E virus infection in pregnancy and maternofoetal outcomes: a systematic review and meta-analysis. BMC Pregnancy Childbirth 2020;20(1):426.
[3] Huang X, Huang Y, Wagner AL, Chen X, Lu Y. Hepatitis E virus infection in swine workers: a meta-analysis. Zoonoses Public Health 2019;66(1):155–163.
[4] WHO: waterborne outbreaks of hepatitis E: recognition, investigation and control. World Health Organization-Technical Report; 2014. p. 1–81.
[5] Ionidis G, Hubscher J, Jack T, Becker B, Bischoff B, Todt D, et al. Development and virucidal activity of a novel alcohol-based hand disinfectant supplemented with urea and citric acid. BMC Infect Dis 2016;16:77.
[6] Feng Z, Lemon SM. Peek-a-boo: membrane hijacking and the pathogenesis of viral hepatitis. Trends Microbiol 2014;22(2):59–64.
[7] Todt D, Friesland M, Moeller N, Praditya D, Kinast V, Bruggemann Y, et al. Robust hepatitis E virus infection and transcriptional response in human hepatocytes. Proc Natl Acad Sci U S A 2020;117(3):1731–1741.
[8] Emerson SU, Nguyen H, Graff J, Stephany DA, Brockington A, Purcell RH. In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. J Virol 2004;78(9):4838–4846.
[9] Meister TL, Klohn M, Steinmann E, Todt D. A cell culture model for producing high titer hepatitis E virus stocks. J Vis Exp 2020;(160).
[10] Todt D, Gisa A, Radonic A, Nitsche A, Behrendt P, Suneetha PV, et al. In vivo evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome. Gut 2016;65(10):1733–1743.
[11] Dremsek P, Wenzel JJ, Johne R, Ziller M, Hofmann J, Groschup MH, et al. Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs. Med Microbiol Immunol 2012;201(2):189–200.
[12] Berto A, Grierson S, Hakze-van der Honing R, Martelli F, Johne R, Reetz J, et al. Hepatitis E virus in pork liver sausage, France. Emerg Infect Dis 2013;19(2):264–266.
[13] Johne R, Trojnar E, Filter M, Hofmann J. Thermal stability of hepatitis E virus as estimated by a cell culture method. Appl Environ Microbiol 2016;82(14):4225–4231.
[14] Schemmerer M, Apelt S, Trojnar E, Ulrich RG, Wenzel JJ, Johne R. Enhanced replication of hepatitis E virus strain 47832c in an A549-derived subclonal cell line. Viruses 2016;8(10).
[15] Rabenau HF, Schwebke I, Blumel J, Eggers M, Rapp I, Steinmann J, et al. Comment on the significance, application and determination of the large volume plating (LVP) : 2. Communication of the DVV/GfV Virus Disinfection Expert Committee on the DVV/RKI Guideline in the version of December 1st, 2014. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 2020;63(5):657–659.
[16] Knegendorf L, Drave SA, Dao Thi VL, Debing Y, Brown RJP, Vondran FWR, et al. Hepatitis E virus replication and interferon responses in human placental cells. Hepatol Commun 2018;2(2):173–187.
[17] Behrendt P, Bremer B, Todt D, Steinmann E, Manns MP, Cornberg M, et al. Significant compartment-specific impact of different RNA extraction methods and PCR assays on the sensitivity of hepatitis E virus detection. Liver Int 2021.
[18] WHO guidelines on hand hygiene in health care: first global patient safety challenge clean care is safer care. edn 2009. Geneva.
[19] Standardization ECf. CEN - EN 1500. 2013. https://standards.globalspec.com/std/1597777/EN%201500.
[20] Suchomel M, Kundi M, Allegranzi B, Pittet D, Rotter ML. Testing of the World Health Organization-recommended formulations for surgical hand preparation and proposals for increased efficacy. J Hosp Infect 2011;79(2):115–118.
[21] Kramer A, Galabov AS, Sattar SA, Dohner L, Pivert A, Payan C, et al. Virucidal activity of a new hand disinfectant with reduced ethanol content: comparison with other alcohol-based formulations. J Hosp Infect 2006;62(1):98–106.
[22] Schielke A, Ibrahim V, Czogiel I, Faber M, Schrader C, Dremsek P, et al. Hepatitis E virus antibody prevalence in hunters from a district in Central Germany, 2013: a cross-sectional study providing evidence for the benefit of protective gloves during disembowelling of wild boars. BMC Infect Dis 2015;15:440.
[23] Siddharta A, Pfaender S, Vielle NJ, Dijkman R, Friesland M, Becker B, et al. Virucidal activity of World Health Organization-recommended formulations against enveloped viruses, including Zika, Ebola, and emerging coronaviruses. J Infect Dis 2017;215(6):902–906.
[24] Steinmann J, Becker B, Bischoff B, Magulski T, Steinmann J, Steinmann E. Virucidal activity of Formulation I of the World Health Organization's alcohol-based handrubs: impact of changes in key ingredient levels and test parameters. Antimicrob Resist Infect Control 2013;2(1):34.
[25] Steinmann J, Becker B, Bischoff B, Paulmann D, Friesland M, Pietschmann T, et al. Virucidal activity of 2 alcohol-based formulations proposed as hand rubs by the World Health Organization. Am J Infect Control 2010;38(1):66–68.
[26] Takahashi M, Tanaka T, Takahashi H, Hoshino Y, Nagashima S, Jirintai Mizuo H, et al. Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: characterization of HEV virions in blood circulation. J Clin Microbiol 2010;48(4):1112–1125.
[27] Yin X, Li X, Feng Z. Role of envelopment in the HEV Life cycle. Viruses 2016;8(8).
[28] European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L: EASL Clinical Practice Guidelines on hepatitis E virus infection. J Hepatol 2018;68(6):1256–1271.
[29] Halbur PG, Kasorndorkbua C, Gilbert C, Guenette D, Potters MB, Purcell RH, et al. Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. J Clin Microbiol 2001;39(3):918–923.
[30] Engle RE, Yu C, Emerson SU, Meng XJ, Purcell RH. Hepatitis E virus (HEV) capsid antigens derived from viruses of human and swine origin are equally efficient for detecting anti-HEV by enzyme immunoassay. J Clin Microbiol 2002;40(12):4576–4580.
[31] Meister TL, Bruening J, Todt D, Steinmann E. Cell culture systems for the study of hepatitis E virus. Antivir Res 2019;163:34–49.