Journal of Virological Methods 263 (2019) 111–119

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Characterization of a quasi-enveloped, fast replicating hepevirus from fish and its use as hepatitis E virus surrogate

Maëlle Bochud[a], Wolfram Schäfer[b], Nathan J. Roth[c], Carlos Ros[a,*]

[a] Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland
[b] CSL Behring GmbH, Emil-von-Behring-Strasse 76, 35041, Marburg, Germany
[c] CSL Behring AG, Wankdorfstrasse 10, 3000, Bern 22, Switzerland

ARTICLE INFO

Keywords:
Cutthroat trout virus
HEV
Virus model
Quasi-enveloped
Hepevirus

ABSTRACT

Hepatitis E virus (HEV) is an emerging concern for the safety of plasma-derived medicinal products. The lack of an efficient cell culture system hampers the studies on HEV biology as well as validation studies to test the capacity of virus reduction steps to clear HEV. Hence, a surrogate hepevirus that can efficiently replicate in cell culture is needed. Cutthroat trout virus (CTV) is a non-pathogenic fish hepevirus, which can replicate in cell culture to high titers. Under interferon inhibition, CTV replication reached up to 5×10^7 genome equivalents per µL in 4-5 days. The intracellular CTV progeny was already lipid-associated, suggesting that the envelope is acquired from intracellular membranes. Transmission electron microscopy of purified quasi-enveloped virus revealed exosome-like structures with an average size of 40 nm, in contrast to 27–34 nm for the non-enveloped virus. The quasi-enveloped virus was significantly less infectious than the non-enveloped virus. Assays based on quantitative RT-PCR, immunofluorescence and immunocytochemistry were established to evaluate virus inactivation. Cold ethanol fractionation removed 3.0 log of CTV and pasteurization of human albumin inactivated more than 3.7 log to below the limit of detection. Similar to HEV, virus replication was promoted in the presence of 17β-estradiol, an effect that can contribute to the understanding of the exacerbated virulence of HEV in pregnant women. These results together reveal substantial similarities between the human and fish HEV and validate CTV as a practical virus model to use in some applications for evaluating the HEV reduction capacity of biological manufacturing process steps.

1. Introduction

First identified in 1978 (Khuroo, 1980), hepatitis E virus (HEV) is a common cause of acute hepatitis (Kamar et al., 2017). While in industrialized countries HEV is endemic, in developing countries the infection occurs in epidemic forms. The infections caused by HEV are generally self-limited, however, chronic infections can also occur in immunocompromised patients and the mortality rates can reach up to 30% among infected pregnant women (Pérez-Gracia et al., 2017). HEV infection is an emerging concern in industrialized countries. In particular the virus has raised concern of transmission through blood donation and nucleic acid screening measures are being considered by certain countries. (European Medicines Agency, 2016). The incidence of HEV in blood and plasma donors in industrialized countries varies significantly and is thought to be related to gastronomic preferences (Roth et al., 2017; Al-Sadeq et al., 2017).

HEV has proven difficult to culture *in vitro*, growing to low titers and requiring long incubation times (Okamoto, 2013). HEV strains have been adapted to improve their replication in different types of cells, such as immortalized, primary and stem cells with varying levels of success (Tam et al., 1997; Shukla et al., 2011; Rogee et al., 2013; Helsen et al., 2016). Some strains showed a better replication following changes in their viral genome, such as the KernowC1/p6 strain that contains a small insertion from the human 40S ribosomal protein S17 (Shukla et al., 2011). Other genetic variants with enhanced replication in cell culture have been found to contain sequences of human origin (Nguyen et al., 2012; Lhomme et al., 2014). Infectious clones have also been developed with the aim to improve replication in cell culture and to facilitate studies on HEV biology (Panda et al., 2000; Yamada et al., 2009). Despite the significant progress in developing cell culture systems with the establishment of increasingly permissive cells and the isolation of adapted HEV variants, their experimental complexity and limited efficiency in terms of titer levels and culture time remain a disadvantage. Besides, the use of isolates with genetic modifications,

* Corresponding author.
E-mail address: carlos.ros@dcb.unibe.ch (C. Ros).

https://doi.org/10.1016/j.jviromet.2018.11.002
Received 23 August 2018; Received in revised form 23 October 2018; Accepted 2 November 2018
Available online 03 November 2018
0166-0934/

which can differ from natural isolates, complicates the studies on HEV. Cell culture systems are also required for validation studies aiming at evaluating virus reduction steps during the manufacturing of plasma-derived medicinal products. However, there are a series of drawbacks associated with the use of HEV. Besides the lack of efficient culture systems to evaluate viral safety and optimize novel strategies of virus elimination, the high prevalence of neutralizing antibodies in human plasma may interfere with inactivation and removal assays, such as virus filtration (Hartl et al., 2016; Horvatits et al., 2018). For these reasons, the use of an HEV model that can efficiently replicate in cell culture to high titers would be particularly useful and an excellent support to the studies carried out with human HEV.

HEV-like viruses have been identified from domestic and wild animals (Meng, 2016b). The expanding identification of novel HEV strains from different animal species may offer opportunities to find an appropriate model for human HEV (Spahr et al., 2018). However, these new HEV variants have in common with human HEV a limited replication in cell culture and the risk of transmission to humans has not yet been fully evaluated. In 1988, ten years after the first identification of HEV, a similar virus was isolated from spawning adult trout and was named cutthroat trout virus (CTV) (Hedrick et al., 1994). Subsequently, CTV has been detected in many salmonid populations and it is not associated with any apparent disease (Batts et al., 2011). Based on genome sequence homology and organization, CTV has been classified in the family *Hepeviridae*, which has been further divided into two genera: genus *Orthohepevirus*, including all mammalian and avian HEV isolates; and genus *Piscihepevirus*, including CTV (Smith et al., 2015). Similar to mammal and avian HEV, the fish HEV genome contains three open reading frames; ORF1 encodes a polyprotein for virus replication, ORF2 encodes the capsid protein, and ORF3 encodes a small phosphoprotein (Batts et al., 2011). A remarkable feature of CTV is that it is the only hepevirus that can efficiently replicate in cell culture (Debing et al., 2013; von Nordheim et al., 2016), and similar to human HEV, CTV derived from cell culture is associated with lipids (von Nordheim et al., 2016).

In this study, methods to propagate CTV to high titers and to evaluate virus infectivity within few days have been optimized. Additionally, the virus progeny generated in cell culture has been characterized and virus reduction steps commonly used in manufacturing processes have been evaluated for their capacity to clear CTV. Our results demonstrate that the fish HEV has substantial similarities to human HEV. Accordingly, the fast-replicating, non-pathogenic CTV represents a practical virus model for virus clearance studies and to gain new insights into the understudied biology of hepeviruses.

2. Materials and methods

2.1. Cells and viruses

The rainbow trout gill (RTGill-W1) and the rainbow trout liver (RTL-W1) cell lines were obtained from Sylvie Bony from the University of Lyon, France. The cells were kept in Leibovitz's (L-15) media (Thermo Fisher Scientific, Waltham, MA, USA) with 10% heat-inactivated foetal calf serum (Amimed, London, UK) and 50 U/mL penicillin/streptomycin (Biochrom/Millipore, Berlin, Germany) at 18 °C. CTV (Heenan88 isolate) was obtained from Yannik Debing from the Rega Institute for Medical Research, Department of Microbiology and Immunology, Leuven, Belgium.

2.2. Virus propagation in cell culture

RTGill-W1 cells (8×10^6 to 10^7) were treated (or not) with BX795 (Sigma-Aldrich, St. Louis, MO, USA) in 5 mL of Minimal Essential Media (MEM) (Sigma) supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2% heat-inactivated foetal calf serum, and 50 U/mL penicillin/streptomycin. One hour after, 100 µL of CTV (2×10^6 genome equivalents per µL; geq/µL) were added. Four hours later, 10 mL of media were added. After 24 h, the culture medium was removed, and 20 mL of fresh MEM were added. Seven days post-infection, the supernatant was collected, aliquoted and stored at −80 °C. Cells were washed with PBS and 10 mL of 0% Leibovitz's (L-15) were used to recover intracellular virus by four freeze/thaw cycles. The media was centrifuged to remove cell debris, aliquoted and stored at −80 °C.

2.3. Quantification of CTV RNA

CTV RNA was quantified as previously described. Briefly, the QIAamp Viral RNA Kit (Qiagen) was used to extract CTV RNA. The extracted samples were prepared for quantification using the Luna Universal One-Step RT-qPCR Kit (New England BioLabs, Ipswich, MA). The primers amplify the genomic region between nucleotides 3247 and 3409: (Forward) 5′-GGC AAC CAT CCT CTA CAA ACA C-3′ and (Reverse) 5′-GAT GTC TTG TGG GAG CCT GTA G-3. Serial ten-fold dilutions of *in vitro* transcribed CTV RNA was used as external standard for quantification.

2.4. Virus concentration

Three different methods of virus concentration were compared for their capacity to concentrate viral particles and preserve their infectivity. For ultracentrifugation (UC), 500 µL of CTV supernatant were added on top of 2 mL 12% sucrose and centrifuged at 60'000 × g for 4 h at 4 °C. The pellet was resuspended in 100 µL PBS. For precipitation with polyethylene glycol 6000 (PEG), a solution of 12% PEG 6000, 0.6 M NaCl was prepared and 5 mL of this solution were mixed with 5 mL of CTV in PBS overnight at 4 °C on a rocker. The mix was centrifuged at 20'000 × g for 90 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 100 µL PBS. For concentration by ultrafiltration, Amicon Ultra-4 Centrifugal Filter Units 100 K were used. A total of 4 mL infected cell culture supernatant was centrifuged at 4'000 × g in a swinging bucket rotor for 20 min at 20 °C. The filtrate was discarded and the retained solution (50 µL) was collected in an Eppendorf tube. The infectivity of viruses concentrated with the different methods was assessed. Cells were infected with the same amount of virus diluted 1:100, 1:500 and 1:2'500 for a period of one week. Subsequently, the amount of CTV RNA was evaluated by RT-qPCR.

2.5. Infectivity assays

Three alternative methods based on end-point dilutions were established for virus titration. Serial dilutions of the virus stock were prepared and inoculated onto replicate cell cultures in 96 well plates. The number of wells containing infected cells were determined for each virus dilution using three different approaches. CTV RNA quantification, ORF2 protein detection by immunofluorescence or by immunocytochemistry. Briefly, well plates were seeded with 4×10^4 RTGill-W1 cells per well in Leibovitz's (L-15) media. Cells were infected with fivefold dilution of the stock virus (4×10^6 geq/µL) in 50 µL 2% MEM. One day after the infection, the media was removed, and 200 µL of fresh 2% MEM were added per well. After two weeks, cells were lysed by a freeze/thaw cycle. RNA was extracted from samples (140 µL) by using the QIAamp viral RNA kit (QIAGEN) and quantified by RT-qPCR. The infectious titer was determined by the Spearman–Kärber method and expressed as log $TCID_{50}$/mL.

Alternatively, the infectious titer was quantified by the ORF2 protein detection using immunofluorescence (IF) or immunocytochemistry (ICC). Cells were grown and infected with tenfold dilutions of CTV in 2% MEM. Six days post-infection, cells were washed with PBS, fixed and permeabilized with 40 µL methanol/acetone (1:1) for 3–5 min at −20 °C. The fixative solution was removed, and cells were dried at

room temperature for 10 min and stored at -20 °C or processed directly. Cells were washed with PBS and blocked with PBS containing 10% (v/v) goat serum (10% PBS-GS) (Dako/Agilent Technologies) for 20 min and incubated with primary antibody (1:300; rabbit anti-ORF2) in 2% PBS-GS for 1 h. Following washes with PBS, cells were incubated with goat anti-rabbit Alexa Fluor 488 antibody in 2% PBS-GS for 1 h in the case of IF and with goat anti-rabbit HRP antibody in 2% PBS-GS in the case of ICC. After washing with PBS, DAPI (4′,6-diamidino-2-phenylindole) was used to stain nuclei for IF and AEC (3-Amino-9-ethylcarbazole) substrate (Sigma) was added to reveal the signal in ICC. After final washings with PBS, 100 µL of glycerol-PBS solution (1:1) were added and the cells were examined with a Zeiss LSM 880 confocal microscope.

2.6. Iodixanol density gradient centrifugation

Layers from 5 to 40% (w/v) OptiPrep™ density gradient medium (Sigma-Aldrich) in L-15 media were placed in Ultra-Clear tubes (14 × 89 mm, Beckman Coulter, Brea, CA). Cell culture supernatant (SN) was added onto the gradient and centrifuged with a TLS55 SN2014 rotor in an Optima XPN-80 ultracentrifuge (Beckman Coulter) at 42'000 rpm for 75 min, for small volumes, and in a SW-41 Ti rotor (Beckman Coulter) at 35'000 rpm at 4 °C for 150 min, for large volumes. Fractions of 100 µL for small volumes and 500 µL for large volumes were collected from the top. The refractive index of each fraction was measured at 4 °C and the RNA concentration was quantified by one-step RT-PCR after RNA extraction. The density, calculated from the refractive index, was plotted with the genome equivalents of each fractions. The fractions corresponding to the peaks were collected and purified with PD-10 desalting columns (Sigma). Virus fractions were stored frozen in 0% MEM.

2.7. Transmission electron microscopy

To facilitate the adsorption of virus particles to the carbon coated grid, one drop of 0.1% of poly-L-lysine was added to a carbon coated grid for 1 min and the excess was removed with filter paper. After the incubation, 5 µL of purified non-enveloped (4×10^7 geq/µL) and quasi-enveloped (1.7×10^7 geq/µL) CTV were deposited on a parafilm in a humid chamber, grids were then placed face down on the virus drops and incubated for 6 h at room temperature. The grids were washed, stained with uranyl acetate and examined using a Philips CM 12 transmission electron microscope equipped with a 12-megapixel Morada camera.

2.8. Virus reduction studies

Effect of cold ethanol fractionation on CTV removal. CTV was mixed with 95% ethanol on ice to obtain a 22% ethanol solution and then added to fraction IV-1 filtrate from the Kistler-Cohn plasma fractionation process at a temperature of −5 °C resulting in a 1:89 dilution of the original CTV stock. Ethanol (95%) was added to a final concentration of 43% ethanol to obtain human derived fraction IV-4 suspension and subsequently the precipitate was removed by depth filtration to obtain fraction IV-4 filtrate. The CTV content in the spiked starting material, the resuspended filter retentate and the final fraction IV-4 filtrate was determined by RT-qPCR.

Effect of pasteurization on CTV inactivation. CTV was spiked into human albumin solution (Alburex®; with a final albumin concentration of 24%), PBS or caprylate/tryptophan (C/T; 20 mM) to get a final virus concentration of 4×10^6 geq/µL. The spiked solutions were heated at 60 °C for ten hours. Intermediate samples were collected at increasing timepoints. After heat treatment, 4×10^4 RTGill-W1 cells in 96-well plates were infected in 50 µL 2% MEM per well. As controls, untreated CTV was resuspended in albumin, PBS or caprylate/tryptophan solutions. One day after the infection, the media was removed and 200 µL of fresh 2% MEM were added per well. After two weeks, the plates were frozen to lyse the cells, RNA was extracted, and CTV RNA was quantified by RT-qPCR, as specified above. The infectious titer was determined by the Spearman–Kärber method and expressed as log $TCID_{50}$/mL.

2.9. RNase assay

A volume of 8 µL of CTV stocks (10^8 geq/µL) were spiked into 2.1 mL of 25% human albumin, PBS or C/T. Aliquots of the samples were heated at 60 °C for increasing times from 0 min to 120 min. After the heat treatment, the samples were place on ice and RNase A (1 µg/µL) was added and incubated at 24 °C for 15 min. Following the treatment, the RNA was extracted, and CTV RNA was quantified by RT-qPCR.

2.10. 17β-estradiol treatment

RTGill-W1 (10^6 cells) were grown in 6-well plates with Leibovitz's media. Media was removed and 17β-estradiol (Sigma) was added in 1 mL of 2% MEM at 0.1 µM, 0.5 µM and 5 µM for 1 h. Cells were infected with CTV (5×10^7 geq/µL) for four hours, washed twice with PBS, and 3 mL of fresh 2% MEM containing 17β-estradiol was added. Five days later, CTV RNA was quantified in the supernatant by RT-qPCR. For kinetics, 140 µL of supernatant and intracellular virus were collected every day until the day 5, extracted and quantified by RT-PCR.

3. Results

3.1. Generation of high titer CTV stocks

RTGill-W1 and RTL-W1 cell lines have been previously shown to be permissive to CTV infection (von Nordheim et al., 2016). As shown in Fig. 1A, CTV reaches peak titer levels of 10^7 geq/µL in RTGill-W1 and 10^6 geq/µL in RTL-W1 within 6 days in culture. During the replication

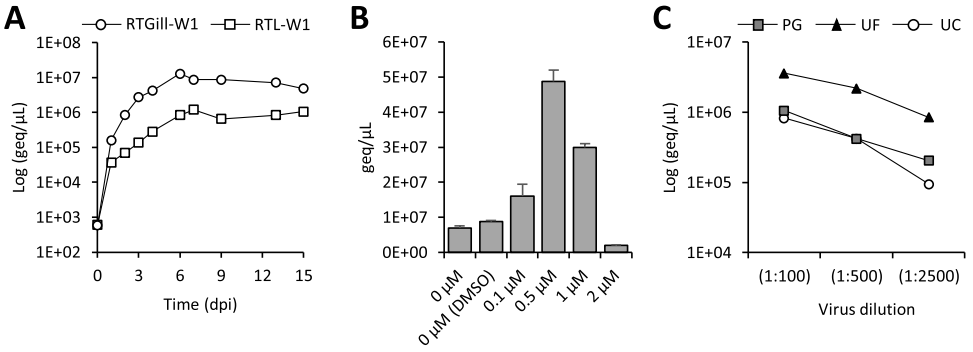


Fig. 1. Generation of high-titer CTV stocks in cell culture and virus concentration. (A) Replication kinetics of CTV in RTGill-W1 and RTL-W1 cell lines. (B) Effect of interferon inhibition on CTV replication. RTGill-W1 cells were treated with increasing doses of BX795. Five days post-infection (dpi), the amount of CTV RNA was quantified. As controls, cells were untreated or treated with DMSO. (C) Effect of virus concentration methods on CTV infectivity. Following virus concentration, cells were infected at different MOI and the amount of CTV RNA was determined by quantitative RT-PCR. PEG, polyethylene glycol 6000; UF, ultrafiltration; UC, ultracentrifugation.

process, CTV generates dsRNA intermediates, which trigger the induction of type I interferon (Poynter et al., 2015). The activation of type I interferon antiviral immunity can reduce the replication efficiency of the virus (Kang and Myoung, 2017). With the aim to further improve the cell culture system, virus propagation was examined in the presence of the transcriptional activity of interferon regulatory factor 3 (Clark et al., 2009) and has been shown to improve HEV replication in cell culture (Devhare et al., 2016). In cells treated with BX795, a seven-fold increase in virus yield was observed, reaching 5×10^7 virus per µL in 5 days (Fig. 1B). Doses above 0.5 µM were toxic for the cells and virus replication was affected.

Virus concentration methods have been extensively used in order to obtain sufficient HEV particles for subsequent assays, although their influence in virus infectivity has not yet been tested. In our studies, ultrafiltration, ultracentrifugation and PEG precipitation were tested for their efficacy and influence in virus infectivity. Following the concentration step, the virus yield was determined by quantitative RT-PCR and the infectivity was measured by quantifying the accumulation of viral RNA in the cell culture supernatant. Ultrafiltration was the method that better recovered the infectivity of the virus (Fig. 1C). Ultracentrifugation and PEG precipitation had a similar detrimental effect on virus infectivity.

3.2. Infectivity assays based on viral RNA and ORF2 detection

A $TCID_{50}$ assay based on viral RNA quantification was used to analyze virus infectivity. RTGill-W1 cells (4×10^4) in L-15 medium were added to wells in 96-well plates and incubated for 3–4 days. Cells were infected with serial dilutions of virus stocks (4×10^6 geq/µL). To produce the virus stock, RTGill-W1 cells were infected in the presence of 0.5 µM of BX795. Subsequently, the supernatant was collected and concentrated by ultrafiltration. At 14 days post-infection, CTV RNA was detected and quantified by RT-PCR. The infectious titer, calculated by the Spearman–Kärber algorithm, was 6.27 log $TCID_{50}$/mL (Fig. 2A). Considering that RNA extraction and quantification is expensive and time-consuming, alternative methods based on ORF2 protein detection by immunofluorescence (IF) and immunocytochemistry (ICC) were also established. An antibody against ORF2 of CTV (von Nordheim et al., 2016), detecting both native and denatured capsids proteins was used (Fig. 2B). Cells in 96-well plates were infected with serial dilutions of virus stocks (10^7 geq/µL) and incubated for 6 days. Following infection, the cells were analyzed by IF and ICC. Similar virus titers of 5.17 and 4.95 log $TCID_{50}$/mL were obtained by IF and ICC, respectively (Fig. 2C).

3.3. Characterization of CTV generated from cell culture

Following infection of RTGill-W1 cells, intracellular and supernatant fractions were subjected to iodixanol gradient ultracentrifugation. Three freeze/thaw cycles were performed to release viruses from intracellular compartments. The presence of CTV in the different iodixanol fractions was determined by quantitative RT-PCR. The results showed that non-enveloped and quasi-enveloped CTV coexist in the intracellular and supernatant fractions (Fig. 3A). While in the supernatant fraction, the non-enveloped CTV is predominant, in the intracellular fraction the major population consists of quasi-enveloped particles. Pre-treatment of intracellular and supernatant fractions with NP40 removed the lipid envelope resulting in a unique population of non-enveloped virus (Fig. 3B). When the fractions were pre-treated with NP40 and pronase, no further shift in density was observed, suggesting that ORF3 was already removed with NP40 alone (Fig. 3C). Similar results were obtained with different concentrations of pronase or with the use of trypsin and chymotrypsin (data not shown).

The presence of non-enveloped CTV population in the cell culture supernatant was intriguing. This virus population could originate from the degradation/removal of the lipids associated with the particles or from an alternative virus egress not involving lipid association. The envelope could be degraded due to freeze/thaw cycles or due to the mild acidic conditions typically found in the cell culture supernatant during virus replication. As shown in Fig. 3E, three freeze/thaw cycles or exposure to mild acidic conditions did not disturb the quasi-enveloped particles.

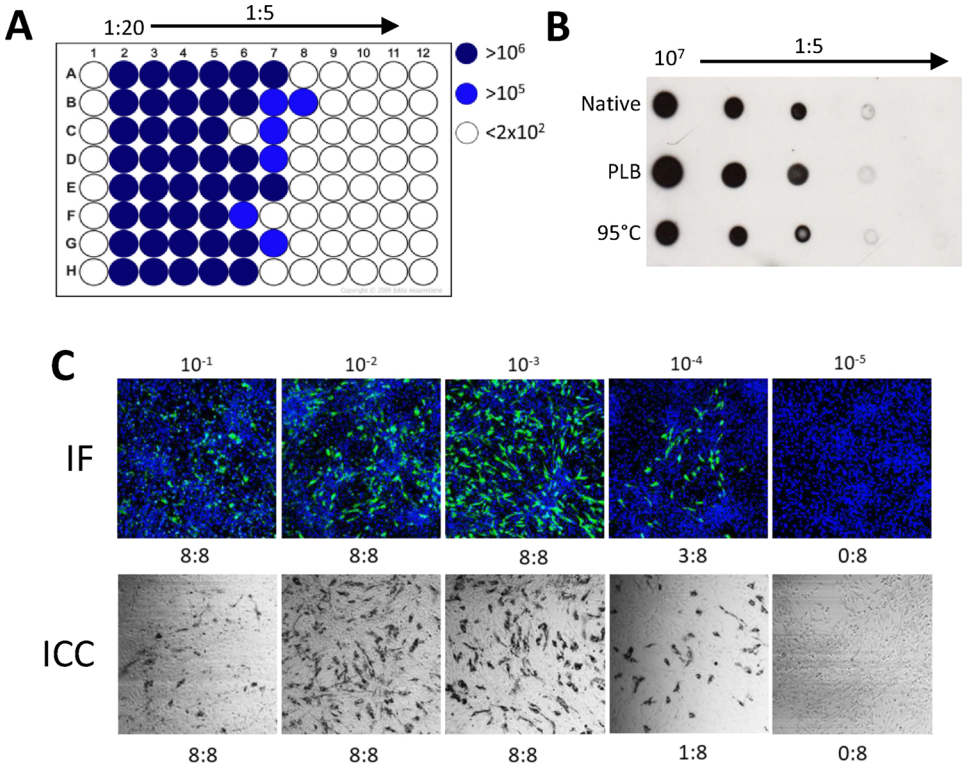
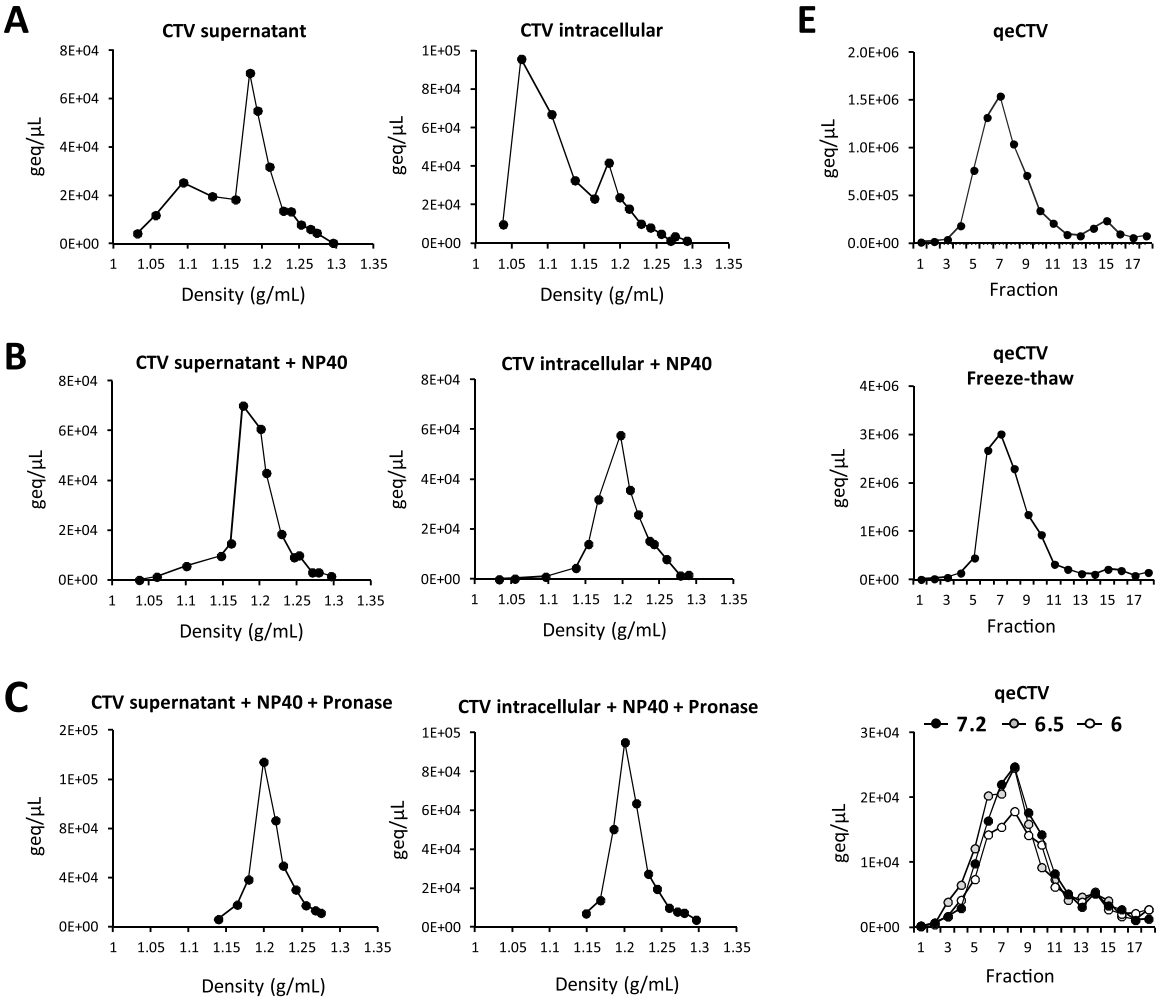


Fig. 2. CTV infectivity assays. (A) $TCID_{50}$ RT-PCR assay. Cells in 96-well plates were infected with serial dilutions of virus stocks (4×10^6 geq/µL) in eight replicates. After 14 days, the amount of CTV RNA was determined by quantitative RT-PCR. The infectious titer was calculated by the Spearman–Kärber method. (B) Detection of native and denatured (PLB; protein loading buffer) CTV capsids by dot blot with an antibody against the ORF2 protein. (C) $TCID_{50}$ IF and ICC assays. Cells in 96-well plates were infected with serial dilutions of virus stocks (10^7 geq/µL) in eight replicates. After 6 days, ORF2 capsid proteins was detected by immunofluorescence (IF) or by immunocytochemistry (ICC) with an antibody against the ORF2. The infectious titer was calculated by the Spearman–Kärber method. The dilutions of the virus and the number of positive wells are indicated (number positive wells:number of replicates).



D

Iodixanol	qeCTV	qeCTV + NP40	qeCTV + NP40 + Pronase
g/mL	1-07-**1.08**	1.17-**1.20**-1.21	1.17-**1.20**-1.21
Shift (g/mL)		0.12	0.12

Fig. 3. Characterization of CTV progeny generated in cell culture. (A) Separation of non-enveloped and quasi-enveloped CTV (qeCTV) from supernatant (SN) and intracellular samples. After iodixanol gradient centrifugation, fractions containing the virus were detected and quantified by RT-qPCR. CTV genome equivalents of each fraction were plotted with density. (B) Iodixanol gradient centrifugation of SN and intracellular virus treated with NP40 (0.1%). (C) Iodixanol gradient centrifugation of SN and intracellular virus treated with NP40 (0.1%) and pronase (0.1%). (D) Buoyant density shift of qeCTV upon treatment with detergent alone and detergent plus pronase (E) Effect of freeze/thaw cycles and exposure to mild acidic conditions on envelope stability. CTV genome equivalents of each fraction are shown.

3.4. Differential structure and infectivity of non-enveloped and quasi-enveloped CTV

CTV non-enveloped and quasi-enveloped particles were separated by iodixanol density centrifugation and examined by transmission electron microscopy. CTV non-enveloped appeared as spherical particles with a diameter ranging from 27 to 34 nm. The quasi-enveloped particles appeared as exosome-like structures, mostly spherical or egg-shaped with an average diameter of 40 nm (Fig. 4A). Similar diameters were reported for HEV (Nagashima et al., 2017). The quasi-enveloped CTV particles were individually enclosed by the lipid membrane, resembling a typical enveloped virus. Lipid structures containing more than one capsid, similar to those revealed in hepatitis A or C viruses (Feng et al., 2013; Ramakrishnaiah et al., 2013), were not observed.

During the course of the infection, four populations of progeny CTV can be detected, i.e., non-enveloped intracellular and extracellular and quasi-enveloped intracellular and extracellular (Fig. 3A). The four virus populations were separated by density gradient centrifugation and their infectivity was examined by $TCID_{50}$-IF, as described above. All four populations were infectious, however, the quasi-enveloped population, was approximately one log less infectious than the non-enveloped virus (Fig. 4B and C).

3.5. Virus reduction by cold ethanol fractionation and pasteurization

In the plasma fractionation process for manufacture of albumin fraction IV-4 precipitation is part of a series of cold ethanol precipitations with increasing ethanol concentrations. It takes place at an alcohol concentration around 40% and removes accompanying proteins from the albumin containing fraction IV-4 filtrate. At this precipitation step CTV clearly partitioned into the precipitate leading to a mean reduction of the virus load in the fraction IV-4 filtrate by 3.0 log (Fig. 5A).

The accessibility of the viral RNA to RNase was used to evaluate the effect of heat on CTV capsid integrity. CTV resuspended in human

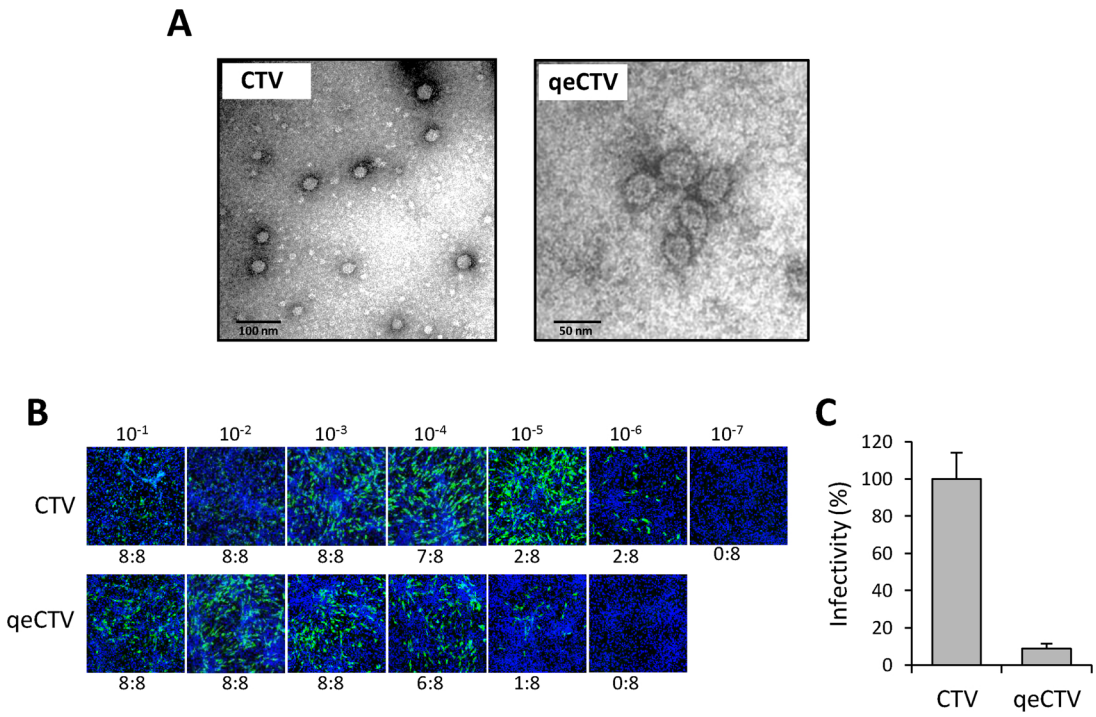


Fig. 4. Differential structure and infectivity of non-enveloped and quasi-enveloped CTV. (A) Transmission electron micrographs of purified CTV and qeCTV derived from cell culture. (B) Infectivity of CTV and qeCTV. Cells in 96-well plates were infected with serial dilutions of purified CTV and qeCTV in eight replicates. After 6 days, the ORF2 protein was detected by immunofluorescence. The dilutions of the virus and the number of positive wells are indicated (number positive wells:number of replicates). (C) Infectious titer of CTV and qeCTV calculated by the Spearman–Kärber method.

serum albumin (HSA; 24%), PBS or caprylate/tryptophan (C/T; 20 mM), used as thermal stabilizer of albumin, was treated at 60 °C for increasing times up to 120 min. Following treatment, the sensitivity of the viral RNA to RNase was evaluated by quantitative RT-PCR. A log reduction factor (LRF) in capsid integrity of 0.95 was observed after 120 min in the presence of HSA. CTV capsids were more sensitive to heat in PBS (1.56 LRF) and in C/T solution (1.79 LRF) (Fig. 5B).

The RNase assay can only measure capsid integrity, which does not necessarily reflect infectivity. To evaluate the effect of heat on CTV infectivity, following heat treatment at 60 °C for increasing times, the infectivity of CTV was evaluated by the $TCID_{50}$/RT-PCR assay. In 24% HSA, CTV was inactivated to below the LOD within 30 min, with a mean RF of ≥ 3.7 log $TCID_{50}$/mL. Similar inactivation was observed when the virus was resuspended in PBS or in C/T solution (Fig. 5C).

3.6. Estradiol treatment stimulates CTV replication

Mortality rates can increase up to 30% among infected pregnant women (Pérez-Gracia et al., 2017) and is thought to be a consequence of the particular hormonal and immunological status during pregnancy (Krain et al., 2014; Navaneethan et al., 2008). In line with this assumption, HEV replication has been recently shown to be enhanced in the presence of 17β-estradiol (Yang et al., 2018). CTV has been isolated only during spawning (Batts et al., 2011) when sex hormone concentrations in plasma are high. Accordingly, it is possible that CTV replication is also exacerbated in the presence of 17β-estradiol. In order to explore this possibility, RTGill-W1 cells were treated with increasing doses of 17β-estradiol, from 0.1 μM, which is near the physiological concentrations in the trout (Espinosa et al., 2013), up to 5 μM. After 5 days in culture, 17β-estradiol resulted in the stimulation of CTV replication (2.11 ± 0.38 and 2.32 ± 0.26 fold, at 0.1 μM and 0.5 μM, respectively). Increased cytotoxicity was observed at doses above 5 μM (Fig. 6A). An increased accumulation of CTV RNA inside the cells and in the cell culture supernatant was observed in the presence of 17β-estradiol over a period of five days (Fig. 6B and C).

4. Discussion

Despite the importance and the global impact of HEV as a human pathogen, the replication mechanism of the virus is still largely unexplored. The main reason of the poor characterization of human HEV is the lack of robust cell culture systems to study the infection in detail. Although significant progress has been made, the virus titers produced in cell culture are typically low and require extensive incubation times and complex experimental settings to mimic the liver environment (Okamoto, 2013). The discovery of novel HEV species from wild and domestic animals (Meng, 2016a), and recently from insects (Wu et al., 2018) should expand the opportunities for developing useful virus models for human HEV. However, the biology and zoonotic potential of these novel animal HEV are still poorly characterized. Besides, the *in vitro* replication of these novel HEV variants is not better than human HEV. An exception to this situation is cutthroat trout virus (CTV), a fish HEV which is non-pathogenic for humans or animals (Batts et al., 2011) that has been shown to replicate efficiently in cell culture (von Nordheim et al., 2016). For this reason, CTV has been proposed as a human HEV surrogate (Debing et al., 2013; von Nordheim et al., 2016). However, the fish HEV is distantly related to human HEV and more data is required to evaluate the degree of similarity between the two viruses. The aims of this study were to improve CTV *in vitro* replication, to characterize CTV progeny generated from cell culture and to evaluate the usefulness of CTV as a human HEV surrogate in virus clearance studies.

We have previously shown that during replication in cultured cells CTV generates dsRNA (von Nordheim et al., 2016) an intermediate RNA species which stimulates strong protective responses, such as the induction of interferon response (Karpala et al., 2005). It has been demonstrated that CTV can induce interferon-like activity in cultures of AK leucocytes (Hedrick et al., 1994). Replication of human HEV was improved by treatment of the cells with the inhibitor BX795, which blocks the expression of interferon-stimulated genes (Devhare et al., 2016). As shown in Fig. 1B, exposure of cells to BX795 resulted in a

A

Sample #	Study #1			Study #2		
	Titre [log_{10}geq/ml]*	Volume [ml]	Load [log_{10}geq]	Titre [log_{10}geq/ml]	Volume [ml]	Load [log_{10}geq]
Spiked starting material (Fr. IV-1 filtrate)	7.7	959.0	10.7	7.6	959.0	10.6
Pool of Fr. IV-4 filtrate and filter postwash	4.6	1453.1	7.8	4.4	1461.7	7.6
Resuspended retentate	8.2	117.7	10.3	7.8	117.2	9.9
Reduction Factor [log_{10}]	**2.9**			**3.0**		

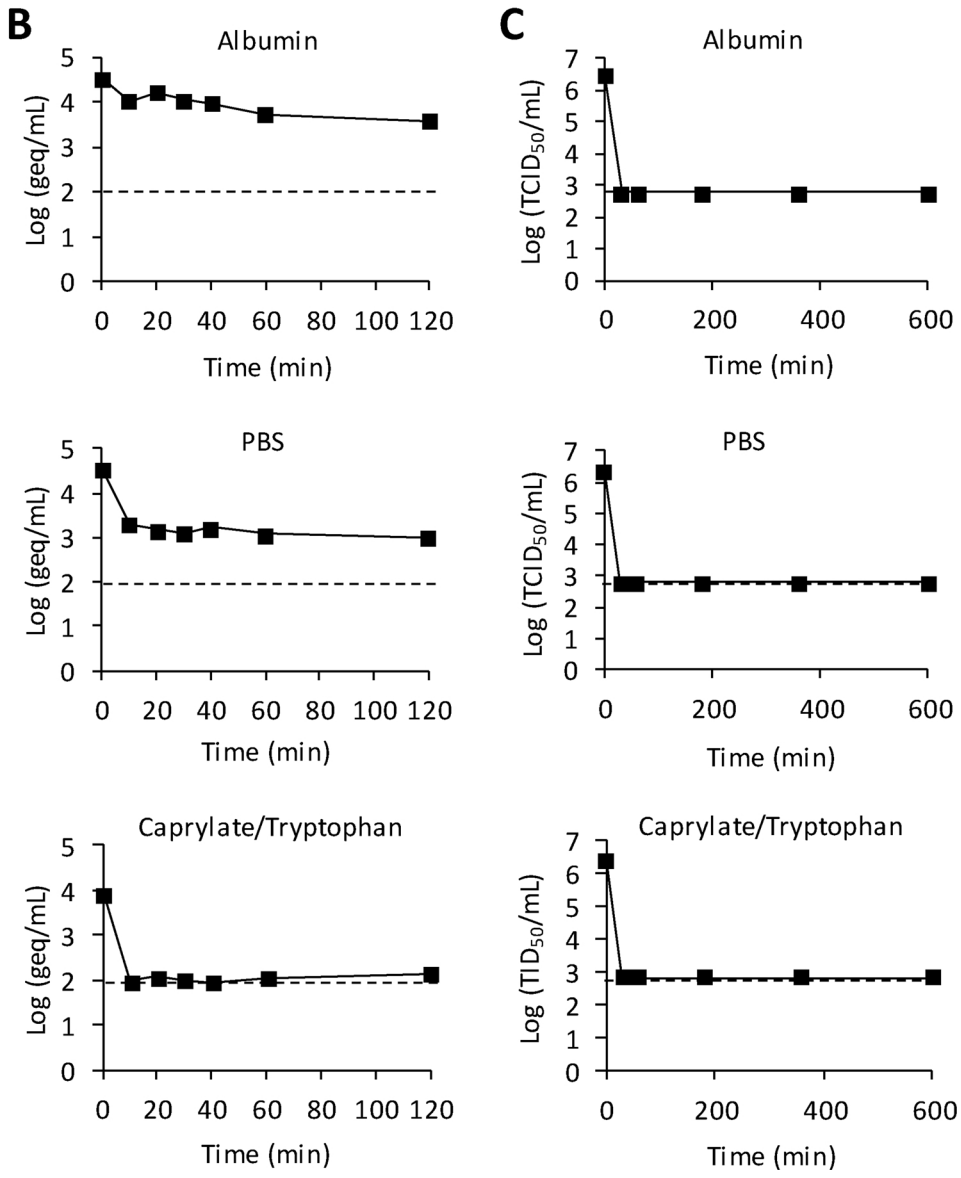


Fig. 5. Virus reduction studies. (A) Partitioning of CTV by cold ethanol fractionation at 43% (v/v) ethanol concentration. Fraction IV-1 filtrate was spiked with CTV and then processed to obtain the albumin containing fraction IV-4 filtrate. Samples were drawn at the beginning (spiked starting material) and the end of the process (Pool of Fr. IV-4 filtrate and filter postwash) and from the waste fraction (Resuspended retentate). Virus content of samples was quantified by RT-qPCR with virus concentration expressed as geq/mL. Reduction factors were calculated as difference of virus loads of starting sample and final sample of the process step. (B) Effect of heat treatment on CTV capsid integrity. The virus was spiked in 25% albumin solution (Alburex®), PBS or caprylate/tryptophan (C/T) and heated at 60 °C for increasing times until 120 min. Following heat treatment, the samples were treated with RNase, extracted and quantified by RT-PCR. The limit of detection (LOD) is shown (discontinued line). (C) Effect of heat treatment on CTV infectivity. The virus was spiked in 25% albumin solution (Alburex®), PBS or C/T and heated at 60 °C for increasing times until 10 h. CTV infectious titer was determined by the $TCID_{50}$ RT-PCR assay. The LOD is shown (discontinued line).

significant improvement of CTV replication, reaching 5×10^7 geq/μL in only 5 days. Replication of human HEV in cell culture is slower and does not reach these high titers. Accordingly, methods of virus concentration such as ultracentrifugation, PEG precipitation and ultrafiltration are frequently required to improve virus yield or to purify viruses from clinical samples. However, the effect of concentration methods on virus infectivity has not yet been evaluated. Our results with CTV suggest that concentration by ultrafiltration results in better recovery of the virus infectivity than ultracentrifugation or PEG precipitation.

Similar to human HEV (Qi et al., 2015), the fish HEV derived from cell culture is associated with lipids (von Nordheim et al., 2016).

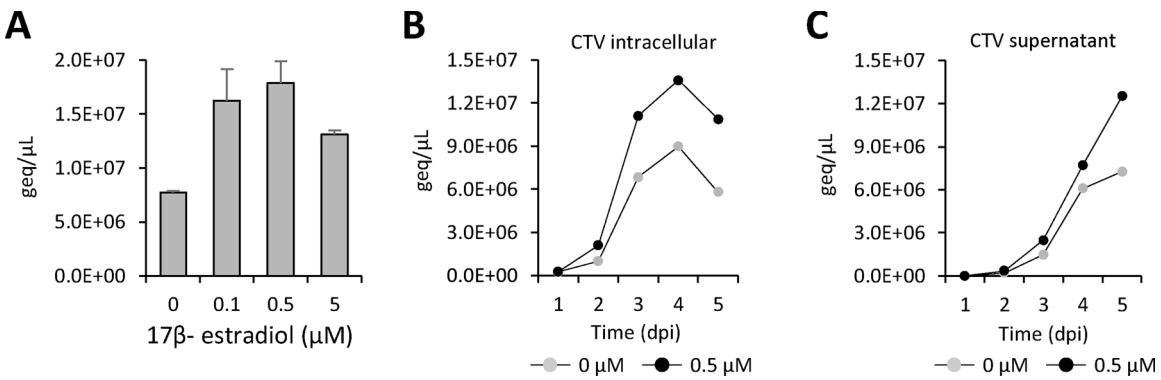


Fig. 6. Effect of 17β-estradiol on CTV replication. (A) Replication of CTV in cells treated with increasing doses of 17β-estradiol. After 5 days, the amount of CTV RNA was quantified by RT-PCR. (B) Effect of 17β-estradiol on the accumulation of intracellular CTV (C) Effect of 17β-estradiol on the accumulation of extracellular CTV.

Intracellular CTV progeny was already associated with lipids and shared the same buoyant density as the extracellular lipid-associated CTV (Fig. 3A). The presence of intracellular quasi-enveloped particles suggests that the acquisition of the envelope occurs most likely following budding of assembled particles into intracellular vesicles, as it has been proposed for human HEV (Nagashima et al., 2014). Detergent treatment efficiently removed the envelope of CTV and further treatment with pronase did not change the buoyant density of the virus. Similar results were obtained with other proteases such as trypsin and chymotrypsin (data not shown), suggesting that ORF3 is incorporated into the lipid membrane and it is efficiently removed by detergent treatment (Fig. 3B, C and D). ORF3 from human HEV has been shown to be integrated into the lipid envelope and to function as an ion channel (Ding et al., 2017).

Non-enveloped and quasi-enveloped particles are found both inside the cell and in the cell culture supernatant. All these virus populations were infectious, but the quasi-enveloped virus was approximately one log less infectious than the non-enveloped virus (Fig. 4). While the intracellular non-enveloped particles are likely assembled progeny particles before vesicle budding, the origin of the extracellular non-enveloped particles is uncertain. The presence of non-enveloped particles in the cell culture supernatant was also reported in human HEV (Qi et al., 2015). A possible explanation would be the degradation of the envelope after virus egress. However, exposure of quasi-enveloped particles to freeze/thaw cycles or to mild acidic conditions similar to those observed in infected cell culture supernatant, did not destabilize the envelope (Fig. 3E). Other explanations might be the interaction of the quasi-enveloped virus with factors on host cellular membranes or an alternative lipid-independent egress mechanism.

CTV has been proposed as a human HEV surrogate to study virus reduction steps in manufacturing processes (EMA, 2016). In a scale down model for a plasma fractionation step in the manufacture of albumin concentrate, cold ethanol fractionation at 43% ethanol removed 3.0 log of CTV. This is in good agreement with 2.6 log HEV removal, which was obtained in a virus spiking study on the same process step (unpublished data).

The effect of heat on CTV capsid integrity and infectivity was evaluated by the RNase assay and by the $TCID_{50}$/RT-PCR assay, respectively. The presence of HSA had a significant stabilizing effect on CTV integrity, similar to that observed in HEV evaluating loss of infectivity (Yunoki et al., 2016). However, when measuring infectivity, CTV was inactivated rapidly, reaching the LOD already at the first measurement point (30 min), with a mean LRF of $\geq$ 3.7 log $TCID_{50}$. In comparison, heat treatment in 12.5% HSA at 58 °C inactivated human HEV to below the LOD within 60–180 min, with a mean LRF of more than 3.1–3.7 log $TCID_{50}$ (Farcet et al., 2016). The faster heat inactivation of CTV compared with HEV can be explained by the fact that the fish HEV replicates at lower temperatures (18 °C). Accordingly, CTV is not an appropriate HEV model for thermal inactivation studies. Progressive adaptation to replicate at 37 °C may increase viral thermostability.

An unexplained phenomenon associated to HEV infection is the increase in mortality rates up to 30% among infected pregnant women (Pérez-Gracia et al., 2017). The explanation for this outcome is not known but hormonal levels during pregnancy are through to play a role (Navaneethan et al., 2008; Krain et al., 2014). In line with this assumption, HEV replication was significantly increased in cells treated with 17β-estradiol (Yang et al., 2018). The increased replication of CTV *in vivo* during spawning and *in vitro* upon treatment of cells with 17β-estradiol (Fig. 6) suggest that CTV infection may also be influenced by changes in sex hormone concentrations and can be useful model to gain insight into this phenomenon.

5. Conclusion

Taken together, our data reveals significant similarities between CTV and HEV and further supports the fish HEV as a useful model for human HEV. The major advantages of CTV as surrogate for HEV are the efficient replication in cell culture, the simplicity of the experimental settings and the lack of biohazard risk, in contrast to human HEV and other more closely related animal HEV species.

Acknowledgements

We thank Sylvie Bony, French National Institute for Agricultural Research, Paris, France, for kindly providing RTGill-W1 and RTL-W1 cell lines and Yannick Debing, University of Leuven, Belgium, for kindly providing cutthroat trout virus. We thank Beat Hänni for his excellent technical assistance with the electron microscopy. We also thank laboratory staff members at CSL Behring Marburg for performing CTV partitioning studies.

References

Al-Sadeq, D.W., Majdalawieh, A.F., Nasrallah, G.K., 2017. Seroprevalence and incidence of hepatitis E virus among blood donors: a review. Rev. Med. Virol. 27https://doi.org/10.1002/rmv.1937. e1937.

Batts, W., Yun, S., Hedrick, R., Winton, J., 2011. A novel member of the family Hepeviridae from cutthroat trout (Oncorhynchus clarkii). Virus Res. 158, 116–123. https://doi.org/10.1016/J.VIRUSRES.2011.03.019.

Clark, K., Plater, L., Peggie, M., Cohen, P., 2009. Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. J. Biol. Chem. 284 (21), 14136–14146. https://doi.org/10.1074/jbc.M109.000414.

Debing, Y., Winton, J., Neyts, J., Dallmeier, K., 2013. Cutthroat trout virus as a surrogate in vitro infection model for testing inhibitors of hepatitis E virus replication. Antiviral Res. 100 (1), 98–101. https://doi.org/10.1016/J.ANTIVIRAL.2013.07.013.

Devhare, P.B., Desai, S., Lole, K.S., 2016. Innate immune responses in human hepatocyte-derived cell lines alter genotype 1 hepatitis E virus replication efficiencies. Sci. Rep.

6, 26827. https://doi.org/10.1038/srep26827.
Ding, Q., Heller, B., Capuccino, J.M.V., Song, B., Nimgaonkar, I., Hrebikova, G., Ploss, A., 2017. Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles. Proc. Natl. Acad. Sci. U.S.A. 114 (5), 1147–1152. https://doi.org/10.1073/pnas.1614955114.
Espinosa, E., Josa, A., Gil, L., Malo, C., Mitjana, O., 2013. Comparing Sex Steroid Levels During the Annual Cycles of Rainbow Trout (*Oncorhynchus mykiss*) Diploid Female (XX) and Triploid Female (XXX) Genotypic Sex. Reprod. Domest. Anim. 48 (1), 149–155. https://doi.org/10.1111/j.1439-0531.2012.02117.x.
Farcet, M.R., Lackner, C., Antoine, G., Rabel, P.O., Wieser, A., Flicker, A., Kreil, T.R., 2016. Hepatitis E virus and the safety of plasma products: investigations into the reduction capacity of manufacturing processes. Transfusion 56 (2), 383–391. https://doi.org/10.1111/trf.13343.
Feng, Z., Hensley, L., McKnight, K.L., Hu, F., Madden, V., Ping, L., Lemon, S.M., 2013. A pathogenic picornavirus acquires an envelope by hijacking cellular membranes. Nature 496 (7445), 367–371. https://doi.org/10.1038/nature12029.
Hartl, J., Otto, B., Madden, R.G., Webb, G., Woolson, K.L., Kriston, L., Pischke, S., 2016. Hepatitis e seroprevalence in Europe: a meta-analysis. Viruses 8 (8). https://doi.org/10.3390/v8080211.
Hedrick, R., LaPatra, S., Yun, S., Lauda, K., Jones, G., Congleton, J., de Kinkelin, P., 1994. Induction of protection from infectious hematopoietic necrosis virus in rainbow trout Oncorhynchus mykiss by pre-exposure to the avirulent cutthroat trout virus (CTV). Dis. Aquat. Org. 20, 111–118. https://doi.org/10.3354/dao020111.
Helsen, N., Debing, Y., Paeshuyse, J., Dallmeier, K., Boon, R., Coll, M., Verfaillie, C.M., 2016. Stem cell-derived hepatocytes: a novel model for hepatitis E virus replication. J. Hepatol. 64 (3), 565–573. https://doi.org/10.1016/J.JHEP.2015.11.013.
Horvatits, T., Ozga, A.-K., Westhölter, D., Hartl, J., Manthey, C.F., Lütgehetmann, M., Pischke, S., 2018. Hepatitis E seroprevalence in the Americas: a systematic review and meta-analysis. Liver Int. https://doi.org/10.1111/liv.13859.
Kamar, N., Izopet, J., Pavio, N., Aggarwal, R., Labrique, A., Wedemeyer, H., Dalton, H.R., 2017. Hepatitis e virus infection. Nat. Rev. Dis. Primers 3, 17086. https://doi.org/10.1038/nrdp.2017.86.
Kang, S., Myoung, J., 2017. Host innate immunity against hepatitis e virus and viral evasion mechanisms. J. Microbiol. Biotechnol. 27 (10), 1727–1735. https://doi.org/10.4014/jmb.1708.08045.
Karpala, A.J., Doran, T.J., Bean, A.G., 2005. Immune responses to dsRNA: implications for gene silencing technologies. Immunol. Cell Biol. 83 (3), 211–216. https://doi.org/10.1111/j.1440-1711.2005.01331.x.
Khuroo, M.S., 1980. Study of an epidemic of non-A, non-B hepatitis: possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. Am. J. Med. 68 (6), 818–824. https://doi.org/10.1016/0002-9343(80)90200-4.
Krain, L.J., Nelson, K.E., Labrique, A.B., 2014. Host immune status and response to hepatitis E virus infection. Clin. Microbiol. Rev. 27 (1), 139–165. https://doi.org/10.1128/CMR.00062-13.
Lhomme, S., Abravanel, F., Dubois, M., Sandres-Saune, K., Mansuy, J.-M., Rostaing, L., Izopet, J., 2014. Characterization of the polyproline region of the hepatitis E virus in immunocompromised patients. J. Virol. 88 (20), 12017–12025. https://doi.org/10.1128/JVI.01625-14.
European Medicines Agency, 2016. Reflection Paper on Viral Safety of Plasma-derived Medicinal Products With Respect to Hepatitis E Virus. Retrieved from. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/06/WC500209354.pdf.
Meng, X.-J., 2016a. Expanding host range and cross-species infection of hepatitis e virus. PLoS Pathog. 12 (8), e1005695. https://doi.org/10.1371/journal.ppat.1005695.
Meng, X.-J., 2016b. Expanding host range and cross-species infection of hepatitis e virus. PLoS Pathog. 12 (8), e1005695. https://doi.org/10.1371/journal.ppat.1005695.
Nagashima, S., Takahashi, M., Jirintai, S., Tanggis, Kobayashi, T., Nishizawa, T., Okamoto, H., 2014. The membrane on the surface of hepatitis E virus particles is derived from the intracellular membrane and contains trans-Golgi network protein 2. Arch. Virol. 159 (5), 979–991. https://doi.org/10.1007/s00705-013-1912-3.
Nagashima, S., Takahashi, M., Kobayashi, T., Tanggis, Nishizawa, T., Nishiyama, T., Okamoto, H., 2017. Characterization of the quasi-enveloped hepatitis e virus particles released by the cellular exosomal pathway. J. Virol. 91 (22), e00822–17. https://doi.org/10.1128/JVI.00822-17.
Navaneethan, U., Al Mohajer, M., Shata, M.T., 2008. Hepatitis E and pregnancy: understanding the pathogenesis. Liver Int. 28 (9), 1190–1199. https://doi.org/10.1111/j.1478-3231.2008.01840.x.
Nguyen, H.T., Torian, U., Faulk, K., Mather, K., Engle, R.E., Thompson, E., Emerson, S.U., 2012. A naturally occurring human/hepatitis E recombinant virus predominates in serum but not in faeces of a chronic hepatitis E patient and has a growth advantage in cell culture. J. Gen. Virol. 93 (Pt 3), 526–530. https://doi.org/10.1099/vir.0.037259-0.
Okamoto, H., 2013. Culture systems for hepatitis E virus. J. Gastroenterol. 48 (2), 147–158. https://doi.org/10.1007/s00535-012-0682-0.
Panda, S.K., Ansari, I.H., Durgapal, H., Agrawal, S., Jameel, S., 2000. The in vitro-synthesized RNA from a cDNA clone of hepatitis E virus is infectious. J. Virol. 74 (5), 2430–2437. http://www.ncbi.nlm.nih.gov/pubmed/10666275.
Pérez-Gracia, M.T., Suay-García, B., Mateos-Lindemann, M.L., 2017. Hepatitis E and pregnancy: current state. Rev. Med. Virol. 27 (3). https://doi.org/10.1002/rmv.1929. e1929.
Poynter, S., Lisser, G., Monjo, A., DeWitte-Orr, S., 2015. Sensors of infection: viral nucleic acid PRRs in fish. Biology 4 (3), 460–493. https://doi.org/10.3390/biology4030460.
Qi, Y., Zhang, F., Zhang, L., Harrison, T.J., Huang, W., Zhao, C., Wang, Y., 2015. Hepatitis e virus produced from cell culture has a lipid envelope. PLoS One 10 (7). https://doi.org/10.1371/journal.pone.0132503. e0132503.
Ramakrishnaiah, V., Thumann, C., Fofana, I., Habersetzer, F., Pan, Q., de Ruiter, P.E., van der Laan, L.J.W., 2013. Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. Proc. Natl. Acad. Sci. U. S. A. 110 (32), 13109–13113. https://doi.org/10.1073/pnas.1221899110.
Rogee, S., Talbot, N., Caperna, T., Bouquet, J., Barnaud, E., Pavio, N., 2013. New models of hepatitis E virus replication in human and porcine hepatocyte cell lines. J. Gen. Virol. 94 (Pt_3), 549–558. https://doi.org/10.1099/vir.0.049858-0.
Roth, N.J., Schäfer, W., Alexander, R., Elliott, K., Elliott-Browne, W., Knowles, J., Simon, T.L., 2017. Low hepatitis E virus RNA prevalence in a large-scale survey of United States source plasma donors. Transfusion 57 (12), 2958–2964. https://doi.org/10.1111/trf.14285.
Shukla, P., Nguyen, H.T., Torian, U., Engle, R.E., Faulk, K., Dalton, H.R., Emerson, S.U., 2011. Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. Proc. Natl. Acad. Sci. U. S. A. 108 (6), 2438–2443. https://doi.org/10.1073/pnas.1018878108.
Smith, D.B., Simmonds, P., Jameel, S., Emerson, S.U., Harrison, T.J., Meng, X.-J., Purdy, M.A., 2015. Consensus proposals for classification of the family Hepeviridae. J. Gen. Virol. 96 (5), 1191–1192. https://doi.org/10.1099/vir.0.000115.
Spahr, C., Knauf-Witzens, T., Vahlenkamp, T., Ulrich, R.G., Johne, R., 2018. Hepatitis E virus and related viruses in wild, domestic and zoo animals: a review. Zoonoses Public Health 65 (1), 11–29. https://doi.org/10.1111/zph.12405.
Tam, A.W., White, R., Yarbough, P.O., Murphy, B.J., McAtee, C.P., Lanford, R.E., Fuerst, T.R., 1997. In VitroInfection and replication of hepatitis e virus in primary Cynomolgus macaque hepatocytes. Virology 238 (1), 94–102. https://doi.org/10.1006/VIRO.1997.8817.
von Nordheim, M., Boinay, M., Leisi, R., Kempf, C., Ros, C., 2016. Cutthroat trout virus-towards a virus model to support hepatitis e research. Viruses 8 (12), 289. https://doi.org/10.3390/v8100289.
Wu, N., Zhang, P., Liu, W., Wang, X., 2018. Sogatella furcifera hepe-like virus: first member of a novel Hepeviridae clade identified in an insect. Virus Res. 250, 81–86. https://doi.org/10.1016/J.VIRUSRES.2018.03.018.
Yamada, K., Takahashi, M., Hoshino, Y., Takahashi, H., Ichiyama, K., Tanaka, T., Okamoto, H., 2009. Construction of an infectious cDNA clone of hepatitis E virus strain JE03-1760F that can propagate efficiently in cultured cells. J. Gen. Virol. 90 (2), 457–462. https://doi.org/10.1099/vir.0.007559-0.
Yang, C., Yu, W., Bi, Y., Long, F., Li, Y., Wei, D., Huang, F., 2018. Increased estradiol in hepatitis E virus infected pregnant women promote viral replication. J. Viral Hepat. 25, 742–751. https://doi.org/10.1111/jvh.12865.
Yunoki, M., Tanaka, H., Takahashi, K., Urayama, T., Hattori, S., Ideno, S., Ikuta, K., 2016. Hepatitis E virus derived from different sources exhibits different behaviour in virus inactivation and/or removal studies with plasma derivatives. Biologicals 44 (5), 403–411. https://doi.org/10.1016/J.BIOLOGICALS.2016.05.004.