Cutthroat trout virus as a surrogate *in vitro* infection model for testing inhibitors of hepatitis E virus replication

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ABSTRACT

Hepatitis E virus (HEV) is one of the most important causes of acute hepatitis worldwide. Although most infections are self-limiting, mortality is particularly high in pregnant women. Chronic infections can occur in transplant and other immune-compromised patients. Successful treatment of chronic hepatitis E has been reported with ribavirin and pegylated interferon-alpha, however severe side effects were observed. We employed the cutthroat trout virus (CTV), a non-pathogenic fish virus with remarkable similarities to HEV, as a potential surrogate for HEV and established an antiviral assay against this virus using the Chinook salmon embryo (CHSE-214) cell line. Ribavirin and the respective trout interferon were found to efficiently inhibit CTV replication. Other known broad-spectrum inhibitors of RNA virus replication such as the nucleoside analog 2'-*C*-methylcytidine resulted only in a moderate antiviral activity. In its natural fish host, CTV levels largely fluctuate during the reproductive cycle with the virus detected mainly during spawning. We wondered whether this aspect of CTV infection may serve as a surrogate model for the peculiar pathogenesis of HEV in pregnant women. To that end the effect of three sex steroids on *in vitro* CTV replication was evaluated. Whereas progesterone resulted in marked inhibition of virus replication, testosterone and 17β-estradiol stimulated viral growth. Our data thus indicate that CTV may serve as a surrogate model for HEV, both for antiviral experiments and studies on the replication biology of the *Hepeviridae*.

KEYWORDS (max 6)

hepatitis E virus, cutthroat trout virus, antiviral, ribavirin, interferon, pregnancy

SHORT COMMUNICATION

Hepatitis E virus (HEV) is one of the leading causes of acute hepatitis worldwide and is transmitted feco-orally (Kamar *et al.*, 2012). Four major genotypes are currently recognized; genotypes 1 and 2 are restricted to humans so far, while viruses in genotypes 3 and 4 are known to be zoonotic agents with their major reservoir in domestic pigs (Kamar *et al.*, 2012). Even though overall mortality among humans is rather low, a recent analysis estimated that genotypes 1 and 2 cause an annual 70 000 deaths and 3000 stillbirths (Rein *et al.*, 2012). Moreover, extraordinary mortality rates up to 25% have been reported in pregnant women, particularly in those infected with genotype 1 (Teshale *et al.*, 2010, Kamar *et al.*, 2012, Hoofnagle *et al.*, 2012). In industrialized countries, symptomatic hepatitis E is seen more frequently in older males (Dalton *et al*., 2011, Davern *et al*., 2011, Mansuy *et al*., 2009). Treatment options are limited, although some experience has been gained in the management of chronic hepatitis E in immune-compromised and transplant patients (Debing *et al.*, 2013). Both pegylated interferon (IFN) alpha and ribavirin (RBV) are effective in most of these patients, but long treatment periods (up to three months) are required and severe adverse effects may occur (Debing *et al.*, 2013). In addition, these regimens are contraindicated in pregnant women. Consequently more effective non-toxic therapeutic options that can be used safely during pregnancy are required.

Studying HEV infection/replication *in vitro* has long been hampered by the lack of susceptible cell culture models. In recent years, such efficient cell culture systems have been reported (Okamoto, 2011, Shukla *et al.*, 2012), but these have not been employed in antiviral studies yet. An alternative approach is to use a surrogate virus, as has been done for example with the duck hepatitis B virus for hepatitis B virus (Nassal *et al.*, 2005) and bovine viral diarrhea virus (BVDV) for hepatitis C virus (HCV) (Buckwold *et al.*, 2003). Cutthroat trout virus (CTV), a small RNA virus of salmonid fish, was proposed as a potential surrogate for HEV (Batts *et al.*, 2011). The CTV genome has a comparable size to the HEV genome and is organized in a similar way (Batts *et al.*, 2011). Nucleotide sequence identity was calculated to be around 40%, with 13 to 26% amino acid identity for the different open reading frames (Batts *et al.*, 2011). Consequently, CTV has been proposed as a potential member of a second genus within the *Hepeviridae* family (Smith *et al.*, 2013). No significant identity was found between the protease domains of CTV and HEV. However, when comparing the amino acid sequences of the helicase and RNA-dependent RNA polymerase of CTV and the 4 different HEV genotypes, 36-39% identity and 52-56% similarity was calculated (BLAST, see also Batts *et al.*, 2011). This argues for a considerably higher degree of evolutionary conservation than for instance the rather poor identity between the polymerase sequences of BVDV and HCV (Choi *et al*., 2004). These observations suggest that CTV could be an interesting model system for testing helicase inhibitors and nucleoside drugs.

CTV was first isolated in 1988 from spawning adult trout and was subsequently detected in many salmonid populations in the western USA (Hedrick *et al.*, 1991, Batts *et al.*, 2011). CTV is considered avirulent in the salmonid species tested to date, but causes a diffuse cytopathic effect (CPE) in Chinook salmon embryo (CHSE-214) cells that is detectable only by visual inspection. We assessed whether this CPE can be quantified by conventional techniques amenable to antiviral assay development such as quantification of intracellular ATP or metabolism of tetrazolium dyes. However, the CTV-induced CPE proved not to be sufficiently extensive. As an alternative, a reverse-transcription quantitative PCR (RT-qPCR)-based virus yield assay was developed. To this end, Nunc 12.5 cm² flasks (Thermo Scientific, Waltham, MA) were seeded with CHSE-214 cells and grown in modified Eagle's medium (MEM; Gibco, Ghent, Belgium) supplemented with 10% fetal bovine serum (FBS; Integro, Zaandam, the Netherlands), 2 mM L-glutamine (Gibco) and 20 mM HEPES (Gibco) at 15°C in sealed flasks. After reaching confluency, cell layers were infected with CTV, based on a protocol described before (Hedrick *et al.*, 1994): culture medium was removed and cell layers were incubated at room temperature with 1mL of MEM with 2% FBS, HEPES (20 mM), penicillin (Gibco, 100 units/mL), streptomycin (Gibco, 100 µg/mL) and CTV at $1.5x10^7$ RNA copies per mL (1988 Heenan lake isolate, GenBank accession number HQ731075). After 24h, the inoculum was removed, cell layers were washed 4 times with 2mL of phosphate-buffered saline, 5mL of fresh medium was added and cultures were incubated at 15°C. As shown in figure 1A, CHSE-214 cells support robust replication of CTV with an overall growth of about 4 orders of magnitude within 2 weeks. So after 14 days, viral RNA was extracted from 150 µL of culture medium (NucleoSpin RNA virus kit, Macherey-Nagel, Düren, Germany) and RT-qPCR of viral RNA was performed with One-Step qRT-PCR mix for SYBR Green I (Eurogentec, Seraing, Belgium) in a final volume of 25 µL containing 125 nM of each primer and 5 µL of RNA sample. The forward (5'-ACTGTTACACCCCATGTAGC-3') and reverse (5'-GGACTTTACTAGCAGTGTGGA-3') primers used in the assay were based on published sequences (Batts *et al.*, 2011). RT-qPCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) under following conditions: 30 min at 48°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 63°C and 30 s at 72°C. Data were analyzed with ABI PRISM 7500 SDS software (version 1.3.1, Applied Biosystems). For absolute quantification, standard curves were generated using 10-fold dilutions of the cloned target cDNA.

Since pegylated IFN alpha and RBV were found to result in some clinical efficacy in (chronically) HEV-infected patients (for review see Debing *et al.*, 2013), the CTV virus yield assay was tested using recombinant rainbow trout IFN 2 (rtIFN2, a generous gift from Jun Zou, University of Aberdeen; Zou *et al.*, 2007) and RBV (Virazole®; ICN pharmaceuticals, Costa Mesa, CA). Viral titers were reduced to 15±9% of virus control for rtIFN2 (20 ng/mL) and to 14±12% and 4±5% for RBV at 1 and 10 µM

respectively ($p<0.001$), without pronounced cytotoxicity (figure 1B), although a slight cytostatic effect could be observed microscopically for RBV at 10 µM. Possible cytotoxicity was assessed in uninfected cultures treated in parallel and assayed for overall metabolic activity by the MTS/PMS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazinemethosulfate) method basically as described (Jochmans et al., 2012). Here, flasks with 2mL of MTS/PMS medium were incubated at room temperature for 3 hours and the optical density (OD) at 498nm was determined for 100µL from each flask.

Next, 2'-*C*-methylcytidine (2'CMC; Carbosynth, Compton, Berkshire, UK) and T705 (favipiravir; BOC Sciences, Shirley, NY), two known nucleoside analog inhibitors of RNA virus replication (Rocha-Pereira *et al.*, 2012, Furuta *et al.*, 2009) were tested for their ability to inhibit growth of CTV. While 2'CMC yielded only modest inhibition of virus replication at 25 and 100 µM (40±30% of virus control, $p<0.05$, and 30±47%, $p=0.06$, respectively, figure 1B), no antiviral activity was observed for T705 at 25 µM (110±61% of virus control, $p=0.79$, $n=3$). Of note, preliminary data from our lab indicate a similar pattern of activity against human HEV in a subgenomic replicon system (Shukla *et al.*, 2012) *in vitro* (Debing, Y., Neyts, J., unpublished results). Taken together, these data suggest that CTV could be used as a surrogate for HEV in antiviral studies. This is especially of interest considering (i) that there is no biohazard risk associated with CTV allowing researchers to easily set up large scale screening efforts, as opposed to human HEV which requires a biosafety level (BSL) 3 environment, and (ii) the robust replication capacity of CTV (see figure 1A) versus the relatively poor growth of HEV in tissue culture.

A potential drawback of the assay as described above is the rather long incubation period. However, in a pilot study to further adapt the virus to cell culture, the incubation period was shortened stepwise to select for fast-replicating strains. After just 9 passages, a comparable increase in viral titers could be reached in 7 days instead of 14 days for the wild type strain (data not shown). With optimized culture conditions and plates, it should be feasible to further down-scale this assay to employ in small-scale screenings. Although the system does not offer a liver-like environment, it provides the possibility to screen for novel candidate inhibitors of HEV replication that are more potent and selective than IFN and RBV. Promising future approaches would be the construction of a selectable replicon expressing a reporter protein, thus allowing easy screening for replication inhibitors, and generation of antibodies for use in sandwich ELISA's, immunofluorescence analyses or for inhibition of viral entry.

In humans, mortality is increased dramatically in pregnant women infected with HEV (Teshale *et al.*, 2010, Kamar *et al.*, 2012, Hoofnagle *et al.*, 2012). This phenomenon is thought to be a consequence

144 of hormonal changes during pregnancy and associated immunologic changes (Navaneethan *et al.*,
145 2008, Pal *et al.*, 2005, Bose *et al.*, 2011). Since CTV is detected only during spawning (Batts *et al.*,
146 2011), it is possible that its replication is hormone-dependent as well. In order to gain a first insight
147 into the possible underlying mechanisms, we studied whether there was a direct effect of sex
148 steroids on CTV replication in CHSE-214 cells. Here progesterone was found to markedly decrease
149 viral titers at 5 and 50 µM (40±26% and 8±5% of virus control, $p<0.05$ and <0.001 respectively, figure
150 2A), whereas testosterone and 17β-estradiol resulted in the stimulation of CTV replication, especially
151 at 0.5 µM (500±480% and 800±600% of virus control, figure 2B). Concentrations of 5 and 50 µM are
152 considerably above physiological levels (e.g. Espinosa *et al.*, 2013). However, CHSE-214 cells are an
153 embryo-derived continuous cell line (Lannan *et al.*, 1984) that may be less sensitive to hormonal
154 stimuli, as was observed for other piscine cell lines (Le Dréan *et al.*, 1995, Fent, 2001), thus requiring
155 increased concentrations of for instance progesterone to obtain notable differences in virus yields. In
156 addition, the maturation-inducing steroid (MIS) in trout is not progesterone, but its 17,20β-
157 dihydroxy-4-pregnen-3-one (17,20β-P) analog (Lubzens *et al.*, 2010). Thus it may be possible that
158 reasonably higher concentrations of the first are required to activate the cognate MIS receptor(s). On
159 the other hand, the stimulation of CTV replication by testosterone and 17β-estradiol was most
160 extensive at 0.5 µM, which is near physiological concentrations (Espinosa *et al*., 2013). Pronounced
161 cytotoxicity was only observed for 17β-estradiol at 50 µM (figure 2B).

162 Trout vitellogenesis (i.e. production of yolk proteins from vitellogenin glycolipophosphoproteins)
163 correlates with an increase in 17β-estradiol concentrations, while 17,20β-P levels remain low
164 (Lubzens *et al.*, 2010, Jalabert, 2005). Subsequently, 17β-estradiol concentrations drop and 17,20β-P
165 concentrations rise during oocyte maturation and ovulation. Similarly, in male fish testosterone
166 concentrations increase during spermatogenesis and decrease at spermiation which again is
167 accompanied by a peak in 17,20β-P levels (Schulz *et al.*, 2010). CTV replication may be influenced
168 directly by these changes in sex hormone concentrations. In conclusion, the notable coincidence
169 between increased replication and shedding of CTV during spawning may not solely be due to
170 hormonal modulation of the host immune system. Another plausible explanation may be growth
171 stimulation of specific cell types by sex steroids (Forsgren and Young, 2012) that are more permissive
172 for viral replication. This could not be studied in our system however. During pregnancy in humans,
173 both progesterone and estradiol levels are elevated. In line with our findings, it was observed that
174 high viral load, decreased expression of the progesterone receptor (PR) and presence of the PROGINS
175 polymorphism in PR are associated with hepatitis E disease severity and poor outcome in pregnancy
176 (Bose *et al.*, 2011). The PROGINS polymorphism is known to diminish response to progesterone
177 (Romano *et al.*, 2007). Thus, it may be hypothesized that decreased progesterone responsiveness

allows for the more vigorous replication of HEV observed. In industrialized regions, symptomatic hepatitis E cases seem to occur more frequently in men over 50 years old (Dalton *et al*., 2011, Davern *et al*., 2011, Mansuy *et al*., 2009). Testosterone levels gradually decrease in older males, which seems in agreement with our findings. Further research is required to study potential pro/antiviral effects of these hormones on HEV replication *in vitro* and *in vivo* and address the underlying mechanisms. However, the somewhat preliminary results obtained in our fish *in vitro* system suggest a possible evolutionary conserved mechanism in the natural history of both CTV and HEV. Moreover for HCV, 17β-estradiol was found to be antiviral at a concentration of 0.4 µM in Huh7.5 cells, but no effect was observed for progesterone (3 µM) (Hayashida *et al*., 2010). This suggests that the replication of multiple hepatitis viruses may thus be directly influenced by sex hormone concentrations.

In conclusion, CTV appears to be an interesting surrogate model for fundamental studies of HEV and for *in vitro* evaluation of potential antiviral drugs. As shown, the CTV replication cycle appears to be influenced by specific hormones, which may prove a useful starting point for future studies into the nature of the high mortality in pregnant women. In addition, CHSE-214 cells were determined to be female with a previously published RT-PCR method (Brunelli *et al.*, 2008) (data not shown). Third, CTV is not pathogenic in humans (or in fish) and consequently it can be used in a BSL1 environment, thus avoiding safety concerns associated with HEV and other more closely related agents such as rat HEV and avian HEV. Finally, salmonid fish such as trout provide a readily available animal model for potential *in vivo* studies (e.g. Hedrick *et al.*, 1994).

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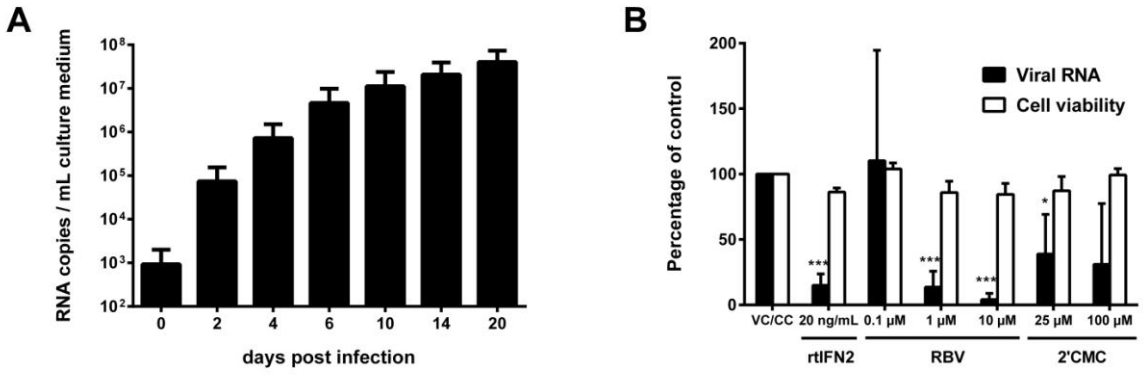
286

FIGURE LEGENDS

Figure 1: A) Kinetics of CTV replication in CHSE-214 cells at 15°C. **B)** Reduction of CTV RNA levels after treatment with rainbow trout interferon 2 (rtIFN2), ribavirin (RBV) or 2'-*C*-methylcytidine (2'CMC) as a percentage of untreated virus control (VC) or uninfected cell control (CC), respectively. Viral RNA copy numbers were normalized to cell counts from similarly treated yet uninfected toxicity controls. Values represent mean ± SD from 3 independent experiments. *** $p<0.001$, * $p<0.05$, unpaired t-test.

Figure 2: Changes in CTV titers and cell viability after treatment with different concentrations of **A)** progesterone, **B)** testosterone or 17β-estradiol as a percentage of virus control (VC) or cell control (CC), respectively. Viral titers were normalized to cell counts as in figure 1. Values represent mean ± SD from at least 2 (cell viability) or 3 (viral RNA) independent experiments. *** $p<0.001$, * $p<0.05$, unpaired t-test.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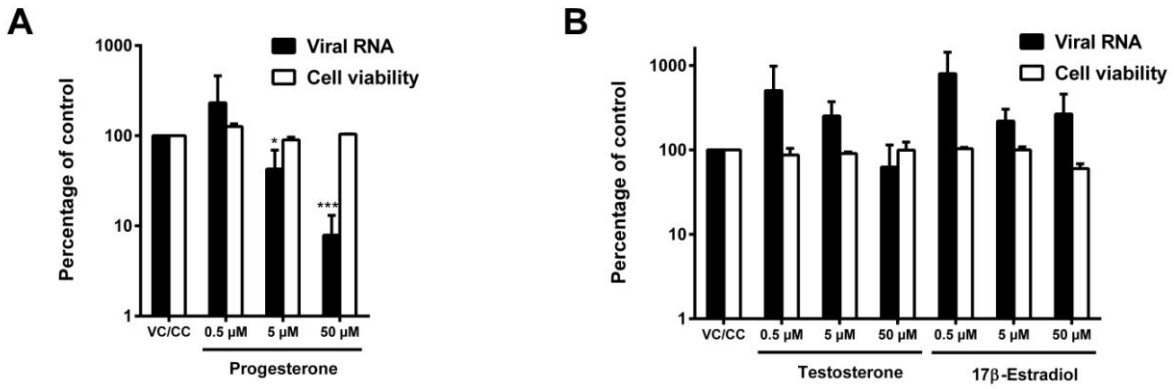
rtIFN2
RBV
2'CMC

Figure 1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50 µM
Testosterone
17β-Estradiol

Figure 2