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A novel member of the family *Hepeviridae* from cutthroat trout (*Oncorhynchus clarkii*)

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ABSTRACT

Beginning in 1988, the Chinook salmon embryo (CHSE-214) cell line was used to isolate a novel virus from spawning adult trout in the state of California, USA. Termed the cutthroat trout (*Oncorhynchus clarkii*) virus (CTV), the small, round virus was not associated with disease, but was subsequently found to be present in an increasing number of trout populations in the western USA, likely by a combination of improved surveillance activities and the shipment of infected eggs to new locations. Here, we report that the full length genome of the 1988 Heenan Lake isolate of CTV consisted of 7269 nucleotides of positive-sense, single-stranded RNA beginning with a 5′ untranslated region (UTR), followed by three open reading frames (ORFs), a 3′ UTR and ending in a polyA tail. The genome of CTV was similar in size and organization to that of *Hepatitis E virus* (HEV) with which it shared the highest nucleotide and amino acid sequence identities. Similar to the genomes of human, rodent or avian hepeviruses, ORF 1 encoded a large, non-structural polyprotein that included conserved methyltransferase, protease, helicase and polymerase domains, while ORF 2 encoded the structural capsid protein and ORF 3 the phosphoprotein. Together, our data indicated that CTV was clearly a member of the family *Hepeviridae*, although the level of amino acid sequence identity with the ORFs of mammalian or avian hepeviruses (13–27%) may be sufficiently low to warrant the creation of a novel genus. We also performed a phylogenetic analysis using a 262 nt region within ORF 1 for 63 isolates of CTV obtained from seven species of trout reared in various geographic locations in the western USA. While the sequences fell into two genetic clades, the overall nucleotide diversity was low (less than 8.4%) and many isolates differed by only 1–2 nucleotides, suggesting an epidemiological link. Finally, we showed that CTV was able to form persistently infected cultures of the CHSE-214 cell line that may have use in research on the biology or treatment of hepevirus infections of humans or other animals.

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1. Introduction

In 1991, a novel RNA virus that was not associated with disease was reported as widespread among trout broodstocks in California, USA (Hedrick et al., 1991). The virus could be isolated using the Chinook salmon embryo cell line, CHSE-214, in which it produced a slow, focal type of cytopathic effect (CPE) that did not result in destruction of the entire monolayer. This cell culture system was used to demonstrate the small, round virus had several characteristics in common with picornaviruses, and that detectable levels of the virus were present at spawning in the ovarian fluids of cutthroat trout (*Oncorhynchus clarkii*), rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*). Termed the cutthroat trout virus (CTV), increased surveillance efforts in subsequent years showed the virus was broadly distributed among trout populations in many areas of the western United States, likely by the widespread shipment of infected eggs to rearing facilities in new locations.

While waterborne exposures of juvenile rainbow trout, cutthroat trout or kokanee salmon (*Oncorhynchus nerka*) to relatively high doses of CTV did not produce mortality, the virus could be re-isolated from some of the animals for 4–6 weeks post-infection (Hedrick et al., 1991). Interestingly, prior exposures of juvenile rainbow trout to CTV provided significant reduction in mortality compared with unexposed controls following laboratory challenges with the fish rhabdovirus, *Infectious hematopoietic necrosis virus*. The increased resistance could be detected for up to 4 weeks post-CTV exposure and was potentially mediated by induction of an interferon response (Hedrick et al., 1994). More

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recently, unpublished epidemiological evidence suggests that CTV may be maintained in a life-long carrier state among some fraction of the population in which the virus is expressed at spawning, possibly due to the immunosuppressive effects associated with sexual maturation.

Hepatitis E virus (HEV) is the type species of the genus *Hepevirus* in the family *Hepeviridae* and has been the subject of several recent and comprehensive reviews (Emerson and Purcell, 2003; Lu et al., 2006; Okamoto, 2007; Panda et al., 2007; Vasickova et al., 2007; Mushahwar, 2008; Purcell and Emerson, 2008; Aggarwal and Naik, 2009; Meng, 2010). The virus is known as the causative agent of outbreaks of either self-limited or fulminant hepatitis in humans and a zoonotic agent that has been detected in a range of mammalian species from many areas of the world. Initially sequenced by Tam et al. (1991), HEV has a single-stranded, positive-sense, capped and poly-adenylated genome of approximately 7.2 kb in length containing three discontinuous and partially overlapping open reading frames coding for a set of structural and non-structural genes flanked by short 5′ and 3′ untranslated regions. Subsequent analysis of HEV sequences from humans and domestic animals revealed the presence of four genotypes that were associated with phylogeographic or epidemiologic differences (Lu et al., 2006; Okamoto, 2007). More recently, a novel HEV genotype was detected in rodents (Johne et al., 2010). The genome of avian hepeviruses shows a similar organization, but the shorter length (6.6 kb) and lower sequence identity (approximately 50% at the nucleotide level) appear sufficiently distinct to support the creation of a separate genus having at least three genotypes (Huang et al., 2004; Bilic et al., 2009; ICTV, 2010; Marek et al., 2010). Here, we report the sequence of the full genome of CTV and show it is a novel member of the family *Hepeviridae*.

2. Materials and methods

2.1. Cell culture and propagation of virus

The 1988 isolate of CTV from adult cutthroat trout spawned at Heenan Lake in Northern California, USA was selected for whole genome sequencing. This isolate represents the index case and was used to provide the initial description of CTV by Hedrick et al. (1991). The Chinook salmon embryo (CHSE-214) cell line (ATCC CRL-1681) was propagated at 15 °C using minimum essential medium as described by Hedrick et al. (1994). To produce stocks of virus for sequence analysis, cultures of CHSE-214 cells were inoculated with CTV at a low multiplicity of infection and the cultures incubated at 15 °C until cytopathic effects (CPEs) were extensive. Aliquots were frozen at −80 °C until used. Cultures of CHSE-214 cells showing CPE following inoculation with the 1988 Heenan Lake strain were photographed using an inverted microscope and aliquots of culture fluid were prepared for electron microscopy as described by Hedrick et al. (1991).

2.2. Sequencing of virus genome

Because the taxonomic affiliation of CTV was initially unknown, genomic sequences of various members of the family *Picornaviridae* were obtained from GenBank and aligned to provide a set of 13 degenerate primers. The primers were used in various combinations for RT-PCR at a low stringency annealing temperature (40 °C) with RNA extracted with TriReagent (Sigma, St. Louis MO) from an aliquot of fluid from a CHSE-214 cell culture infected with CTV. One primer pair produced an amplicon of >300 bp that was suitable for sequencing using Big Dye chemistry and a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The CTV sequence showed the highest similarity to that of *Hepatitis E virus* by BLAST search (up to 40% aa identity). New sets of PCR primers were made using this authentic sequence and the full genome of the 1988 Heenan Lake isolate was obtained by a combination of primer walking and 3′ or 5′ RACE. For the 5′ RACE and 3′ RACE, we used kits (Life Technologies, Carlsbad, CA) according to the manufacturer's protocols with specific primers designed to bind to genomic RNA sequences of the 1988 Heenan Lake isolate of CTV. In addition to kit primers, a primer containing a string of G's followed by either a T, C, or A was used. This polyGT primer allowed sequencing back in the 3′ direction from the 5′ end of the genome to confirm the identity of the terminal nucleotide. Another primer 5′-TGAGCGTTAGTAATTTTTCGTTC-3′ was designed to bind to the 5′ end of the genome allowing confirmation of the 5′ UTR.

2.3. Sequencing of isolates from various hosts and geographic areas

To determine if genetic variation existed among isolates of CTV from different hosts or geographic areas, a panel of 63 virus isolates, presumed to be CTV on the basis of cytopathic effects in CHSE-214 cells, was assembled and stock virus suspensions prepared. Virus RNA was extracted from each isolate with TriReagent and subjected to RT-PCR using primers designed to amplify a portion of ORF1 coding for the CTV polyprotein. Primers and reaction conditions were: (+) primer 5′-ACTGTTACACCTCATGTAGC-3′; (−) primer 5′-GACTTTACTAGCAGTGTGGAT-3′; reverse transcription at 50 °C for 30 min, denaturation at 95 °C for 2 min followed by 30 cycles of: 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The resulting amplicons were sequenced and the sequences trimmed, aligned and analyzed using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI) and MacVector 7.2 (Accelrys, San Diego, CA). The 63 isolates produced 38 unique sequences. A representative isolate was chosen for each and the sequence deposited in GenBank (Table 1).

2.4. Sequence analysis

The nucleotide sequences of the 5′ and 3′ untranslated regions and the nucleotide and deduced amino acid sequences of the three open reading frames of the 1988 Heenan Lake isolate of CTV were compared with those of hepeviruses from human (GenBank M73218), rodent (GenBank GU345043) and avian (GenBank EF206691) sources. In addition, the deduced amino acid sequence for ORF 1 of CTV was compared with ORF 1 sequences of representative calici-, toga- or picornaviruses including: *Rabbit hemorrhagic disease virus*, *Norwalk virus*, *Sapporo virus*, *Vesicular exanthema of swine virus*, *Sindbis virus*, *Rubella virus*, and *Poliovirus*. A phylogenetic analysis was performed using the neighbor-joining and parsimony programs in the PAUP* version 4.0b software package (Swofford, 1998). For this analysis, we compared a 225 aa region of the helicase domain of the 1988 Heenan Lake isolate of CTV with the corresponding regions of representatives of the four genotypes of HEV, rodent and avian hepeviruses, and two members of the family *Togaviridae*, *Sindbis virus* and *Rubella virus*. To determine if various isolates of CTV appeared to cluster by host, geographic area, or year of isolation, a phylogenetic analysis was similarly performed using the 38 unique, partial ORF 1 sequences obtained from the 63 isolates in which bootstrap values below 70 were collapsed.

2.5. Persistently infected cultures

Because epidemiological evidence suggested that CTV might be maintained as an inapparent, but perhaps life-long, carrier state in trout, we attempted to establish persistently infected cultures of CHSE-214 cells. After inoculation of cell cultures, viral CPE was allowed to progress to completion before the cell sheet was rinsed

Table 1
Isolates of cutthroat trout virus (CTV) representing unique sequence types and the GenBank Accession Number for the sequence of the helicase domain corresponding to nucleotide positions 3178–3439 of the CTV genome.

Location	State	Year	Trout host	GenBank no.
Heenan Lake	CA	1988	Cutthroat (*Oncorhynchus clarkii*)	HQ846595
Hot Creek	CA	1988	Rainbow (*Oncorhynchus mykiss*)	HQ846613
Mount Shasta	CA	1988	Brown (*Salmo trutta*)	HQ846610
Roaring River	OR	1989	Rainbow (*Oncorhynchus mykiss*)	HQ846619
Oak Springs	OR	1989	Rainbow (*Oncorhynchus mykiss*)	HQ846590
Richfield	UT	1989	Rainbow (*Oncorhynchus mykiss*)	HQ846598
Darrah Springs	CA	1989	Rainbow (*Oncorhynchus mykiss*)	HQ846612
American River	CA	1990	Rainbow (*Oncorhynchus mykiss*)	HQ846608
Shasta	CA	1990	Rainbow (*Oncorhynchus mykiss*)	HQ846599
Ford	WA	1990	Brown (*Salmo trutta*)	HQ846603
San Joaquin	CA	1991	Brook (*Salvelinus fontinalis*)	HQ846587
Humboldt	CA	1992	Cutthroat (*Oncorhynchus clarkii*)	HQ846623
Mojave	CA	1993	Rainbow (*Oncorhynchus mykiss*)	HQ846609
Deadman	CA	1993	Golden (*Oncorhynchus aguabonita*)	HQ846597
Heenan Lake	CA	2000	Cutthroat (*Oncorhynchus clarkii*)	HQ846615
Mount Shasta	CA	2002	Rainbow (*Oncorhynchus mykiss*)	HQ846604
Crystal Lake	CA	2007	Rainbow (*Oncorhynchus mykiss*)	HQ846614
Glenwood Springs	CO	2008	Rainbow (*Oncorhynchus mykiss*)	HQ846593
Pitkin1	CO	2008	Cutthroat (*Oncorhynchus clarkii*)	HQ846622
Pitkin2	CO	2008	Cutthroat (*Oncorhynchus clarkii*)	HQ846618
Poudre	CO	2008	Cutthroat (*Oncorhynchus clarkii*)	HQ846616
Jackson	WY	2008	Cutthroat (*Oncorhynchus clarkii*)	HQ846607
Jackson226	WY	2008	Cutthroat (*Oncorhynchus clarkii*)	HQ846592
Saratoga	WY	2008	Brown (*Salmo trutta*)	HQ846611
Murray Springs	MT	2008	Rainbow (*Oncorhynchus mykiss*)	HQ846602
Ennis1	MT	2008	Rainbow (*Oncorhynchus mykiss*)	HQ846620
Ennis5	MT	2008	Rainbow (*Oncorhynchus mykiss*)	HQ846606
Ennis6	MT	2008	Rainbow (*Oncorhynchus mykiss*)	HQ846591
Washoe Park	MT	2008	Cutthroat (*Oncorhynchus clarkii*)	HQ846596
Ennis5 brood	MT	2009	Rainbow (*Oncorhynchus mykiss*)	HQ846589
Washoe Park	MT	2009	Cutthroat (*Oncorhynchus clarkii*)	HQ846594
Crystal River	CO	2009	Cutthroat (*Oncorhynchus clarkii*)	HQ846624
Hagerman Valley	ID	2009	Rainbow (*Oncorhynchus mykiss*)	HQ846617
Willow Beach	AZ	2010	Rainbow (*Oncorhynchus mykiss*)	HQ846605
Williams Creek	AZ	2010	Apache (*Oncorhynchus apache*)	HQ846601
Seven Springs	NM	2010	Cutthroat (*Oncorhynchus clarkii*)	HQ846600
Mora1	NM	2010	Gila (*Oncorhynchus gilae*)	HQ846621
Mora5	NM	2010	Gila (*Oncorhynchus gilae*)	HQ846588

and fresh culture medium added to the flasks. The culture fluid was replaced at approximately 2-week intervals as the cell monolayer began to regenerate and aliquots of spent media were stored at −80 °C. Once the cell monolayer had regenerated, the culture was split and propagated as for normal cells. Total RNA was extracted from samples of the culture fluid collected over a 30-week period using TriReagent and the RNA was diluted in water at 0, 1:10, and 1:100 then analyzed by RT-PCR to provide an approximation of the relative CTV concentrations in culture fluids at the selected passages.

3. Results

3.1. Characteristics of the virus

All isolates of CTV grew well in CHSE-214 cell cultures where they produced typical CPE after approximately 14 d of incubation (Fig. 1). Electron microscopy of pelleted cell culture fluid confirmed that CTV was a spherical virion of approximately 31 nm (30.94 ± 0.94) in diameter (Fig. 2), slightly smaller than the 37.5 nm initially reported by Hedrick et al. (1991) but in good agreement with size estimates for virions of other hepeviruses (Purcell and Emerson, 2008).

3.2. Sequencing of virus genome

The full length genome of the 1988 Heenan Lake isolate of CTV (GenBank Accession Number HQ731075) consisted of 7269 nucleotides (nt) of positive-sense, single-stranded RNA beginning with a 5′ untranslated region (UTR), followed by three open reading frames (ORFs), a 3′ UTR and ending in a polyA tail (Table 2). The genome of CTV was very similar to HEV in both size and organization (Fig. 3). Multiple sequence reads were performed in both sense and antisense directions with complete sequence agreement. This was true even for two nt positions in the genome where het-

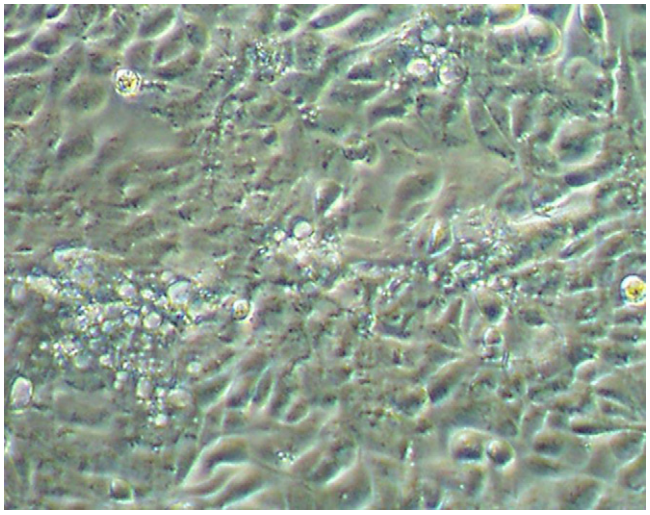

Fig. 1. Cytopathic effect of CTV in a culture of CHSE-214 cells incubated at 15 °C. The virus grows relatively slowly, producing a diffuse type of CPE and incomplete destruction of the monolayer.

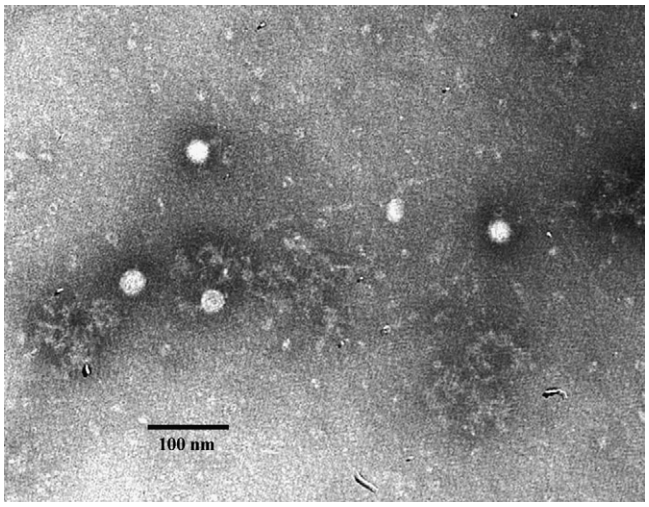

Fig. 2. Electron micrograph of the cutthroat trout virus. The 1988 Heenan Lake isolate of CTV used for sequence analysis was propagated in cultures of CHSE-214 cells and the virus prepared for electron microscopy as described by Hedrick et al. (1991). Negatively stained preparations revealed the small, round virions had a diameter of approximately 31 nm (30.94 ± 0.94). No evidence of other virus-like particles was observed in the preparations.

Table 2
The genome of the cutthroat trout virus (CTV) consists of 7269 nucleotides (nt) of positive-sense, single-stranded RNA organized as three open reading frames (ORFs) flanked by untranslated regions (UTRs) at the 5′ and 3′ends (Fig. 3). The predicted number of amino acids (aa), isoelectric point (pI) and molecular weight (MW) is given for the proteins encoded by each of the three open reading frames (ORFs) identified in the CTV genome. By analogy with other hepeviruses, the large, non-structural polyprotein coded by ORF 1 was shown to contain multiple domains including: methyltransferase, protease, helicase and polymerase while ORF 2 encoded the structural capsid protein and ORF 3 encoded the structural phosphoprotein.

	Start (nt)	End (nt)	Length (nt)	aa	pI	MW
5′ UTR	1	100	100	–	–	–
ORF 1	101	5224	5124	1707	8.4	189,614
ORF 2	5289	7193	1905	634	5.7	68,003
ORF 3	5986	6663	678	225	11.8	24,498
3′ UTR	7194	7269	76	–	–	–

erogeneity was found. At nt position 2851, both T and A peaks were present in the sequence chromatograms (70% T), but both nucleotides result in a codon for the amino acid, isoleucine. At position 5162, both G and C peaks were present (70% G); however, a "G" results in a codon for aspartic acid, while a "C" encodes a basic histidine residue.

By comparison with the genome organization of other hepeviruses, ORF 1 of CTV (nt 101–5224 of the 1988 Heenan Lake isolate) was judged to encode a polyprotein with multiple, non-structural, enzymatic domains including: methyltransferase, protease, helicase and polymerase. The length of ORF 1 of CTV was similar to that of HEV (Table 3) and the calculated isoelectric point was 8.4, higher than that of either avian or human hepeviruses (pI 6.9–7.2). Pairwise alignment of the deduced amino acid (aa) sequence of ORF 1 of CTV with that of mammalian or avian hepeviruses showed an overall amino acid sequence identity of 26–27% (Table 4). Within ORF 1, the methyltransferase domain spanned aa 62–245 where the four signature amino acid residues described by Rozanov et al. (1992) were found at aa 67, 118, 121 and 234, three of which were conserved in CTV, while asparagine replaced aspartic acid at aa 118. The protease domain included aa 430–594 and contained an unusually high number (17 of 27) of serine residues in the region of aa 570–596 that were not present in the protease of HEV. As described for HEV, a proline-rich region (aa 698–725) was present within the proposed hinge region (aa 696–772). The amino acids of domain Y, the putative papain-like cysteine protease, and domain X, first identified by Koonin et al. (1992), were not highly conserved in CTV. The helicase domain of CTV spanned aa 967–1221 and the characteristic amino acid sequence of the nucleotide triphosphate binding site (GVPGSGKS) within the HEV helicase (Panda et al., 2007) was found to be completely conserved at positions 982–989; however, the DEAP motif of the HEV helicase was present in CTV at positions 1039–1042 as DEVF. The RNA-directed RNA polymerase (RdRp) of CTV was located at aa 1224–1707 and the conserved tripeptide motif, GDD, described by Fry et al. (1992) was present at amino acid positions 1572–1574. In addition, other regions within the

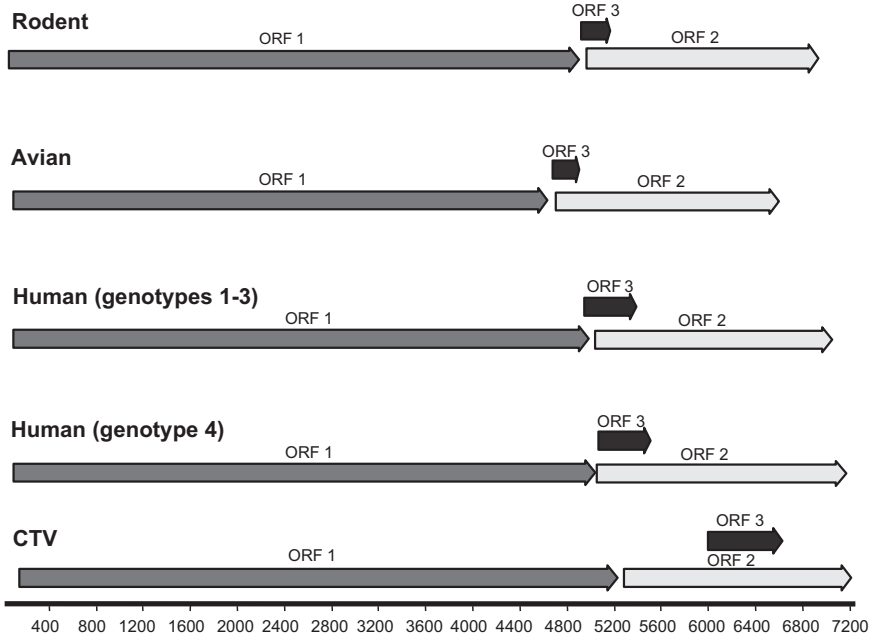

Fig. 3. Genome organization of CTV. The overall genome consisted of 7269 nucleotides of single-stranded RNA organized as three ORFs, similar to that reported for hepeviruses from human, rodent or avian sources.

Table 3
Comparison of the genomes of the 1988 Heenan Lake isolate of CTV (GenBank HQ731075) with that of hepeviruses from avian (GenBank EF206691), human (GenBank M73218) or rodent (GenBank GU345043) sources. The nucleotide (nt) numbers and (length) are indicated for the 5′ and 3′ untranslated regions (UTRs) and the three open reading frames (ORFs) coding for the large non-structural polyprotein, the capsid protein and the phosphoprotein. The predicted isoelectric point (pI) is also shown for each protein.

Virus	Total length (nt)	5′ UTR	Non-structural (ORF 1)	Capsid (ORF 2)	Phosphoprotein (ORF 3)	3′ UTR
CTV	7269	1–100 (100)	101–5224 (5124) pI 8.4	5289–7193 (1905) pI 5.7	5986–6663 (678) pI 11.8	7194–7269 (76)
Avian HEV	6649	1–24 (24)	25–4617 (4593) pI 6.9	4704–6524 (1821) pI 8.8	4651–4914 (264) pI 8.8	6525–6649 (125)
Human HEV	7194	1–27 (27)	28–5109 (5082) pI 7.2	5147–7129 (1983) pI 9.2	5106–5477 (372) pI 12.5	7130–7194 (65)
Rodent HEV	6945	1–10 (10)	11–4921 (4911) pI 8.7	4949–6883 (1935) pI 8.2	4938–5246 (309) pI 6.0	6884–6945 (62)

RdRp of CTV showed a high level of sequence identity with isolates of HEV, such as aa 1480–1496 where CTV was 88% identical and aa 1536–1544 where CTV was completely identical (KKHSGEPGT) with the sequences of both avian and human hepeviruses. Overall, the amino acid sequence of the helicase domain of CTV was 33–35% identical to that of other hepeviruses while the RdRp domain was 34–36% identical.

Open reading frame 2 of CTV (nt 5289–7193 of the genomic sequence of the 1988 Heenan Lake isolate) appeared to encode the structural capsid protein of 634 amino acids (Table 2). The length of ORF 2 of CTV was similar to that of HEV (Table 3). Pairwise alignment of the amino acid sequences of ORF 2 from the 1988 Heenan Lake CTV with avian, rodent or human hepeviruses revealed 18–21% identity (Table 4). The capsid protein of HEV is reported to have a highly basic amino acid content that is assumed to facilitate RNA encapsidation (Panda et al., 2007); however, ORF 2 of CTV had an isoelectric point of 5.7, far more acidic than the pI of 8.8–9.2 reported for avian or human hepeviruses. Many acidic residues were present at the 3′ end of ORF 2 where nine of 18 concluding amino acids were either aspartic acid or glutamic acid.

Open reading frame 3 of CTV (nt 5986–6663 of the genomic sequence of the 1988 Heenan Lake isolate) was analogous to ORF 3 of other hepeviruses suggesting it encoded a phosphoprotein of 225 amino acids (Table 2). This highly basic protein (pI 11.8) was most similar to that encoded by ORF 3 of human HEV (pI 12.5), although the pairwise amino acid identity between the phosphoproteins of the two viruses was low at only 13–16%. The ORF 3 for CTV did not overlap ORF 1 at its 5′ end as in HEV genotypes 1–3 (Fig. 3) and was more similar in organization to HEV genotype 4 (Panda et al., 2007). Instead of the short 5′ UTR typical of mammalian or avian hepeviruses (24–27 nt), the 5′ UTR of CTV was 100 nt in length (Table 2) while the 3′ UTR of 76 nt in length was more typical of other members of the family (65–125 nt).

3.3. Sequence comparisons with other members of the Hepeviridae and related viruses

Pairwise alignments of the deduced amino acid sequence for ORF 1 of CTV with ORF 1 sequences of human, rodent and avian hepeviruses showed amino acid sequence identities of 26–27% (Table 4), significantly higher than the aa identities between CTV and the ORF 1 sequences for representative calici-, toga- or picornaviruses including: *Rabbit hemorrhagic disease virus* (13%), *Norwalk virus* (13%), *Sapporo virus* (13%), *Vesicular exanthema of swine virus* (14%), *Sindbis virus* (13%), *Rubella virus* (16%), or *Poliovirus* (13%). A 225 aa region of the CTV helicase domain (aa 967–1221) was used in a neighbor-joining analysis to construct a phylogenetic tree with the most closely related viruses (Fig. 4). These analyses revealed that CTV was sufficiently distinct from other members of the family *Hepeviridae* to be considered a potential member of a novel genus.

3.4. Sequencing of isolates from various hosts and geographic areas

A 262 nt region of the helicase domain corresponding to position 3178–3439 of the CTV genome was sequenced for 63 isolates of virus obtained from seven species of trout in the western United States. The analysis revealed 38 individual sequence types represented by the isolates listed in Table 1. While the maximum diversity within the 262 nt region analyzed was 8.4%, many isolates differed from the 1988 Heenan Lake isolate by only 1–2 nt, providing data that were difficult to interpret. The isolates did not appear to group by year of isolation or by host species, and a low level of variation was found even among isolates recovered from fish collected at the same facility in the same year. When subjected to phylogenetic analysis, the isolates formed a tree with two major clades, but without strong bootstrap support (Fig. 5). The majority of isolates, including the 1988 Heenan Lake isolate of CTV, formed

Table 4
Percent nucleotide (nt) identity in pairwise alignments for the 5′ and 3′ untranslated regions (UTRs) and percent amino acid (aa) identity for the three open reading frames (ORFs) coding for the large non-structural (NS) polyprotein, the capsid protein and the phosphoprotein of the 1988 Heenan Lake isolate of cutthroat trout virus (CTV, GenBank HQ731075) with that of hepeviruses from avian (AHEV, GenBank EF206691), human (HEV, GenBank M73218) and rodent (RHEV, GenBank GU345043) sources.

Comparison	5′ UTR % nt	Non-structural ORF 1 % nt (% aa)	Capsid ORF 2 % nt (% aa)	Phosphoprotein ORF 3 % nt (% aa)	3′ UTR % nt
CTV vs AHEV	42	43 (26)	38 (21)	43 (14)	46
CTV vs HEV	44	43 (26)	39 (19)	41 (13)	40
CTV vs RHEV	40	43 (27)	39 (18)	45 (16)	49
AHEV vs HEV	46	50 (42)	52 (45)	45 (20)	52
AHEV vs RHEV	50	51 (44)	51 (42)	45 (28)	76
HEV vs RHEV	60	53 (49)	57 (55)	46 (29)	55

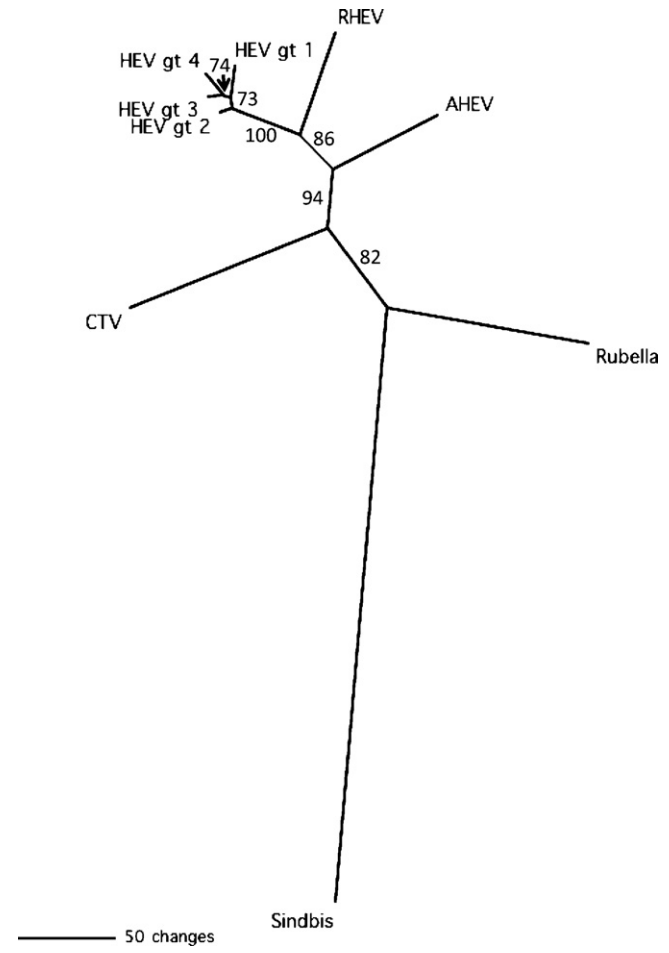

Fig. 4. Phylogenetic analysis of CTV. Sequence comparisons of a 225 aa region of the helicase domain within ORF 1 of the 1988 Heenan Lake isolate of CTV with that of other members of the *Hepeviridae* revealed that CTV was distinct from hepeviruses of avian, rodent or human origins or from two members of the family *Togaviridae*, *Sindbis virus* and *Rubella virus*.

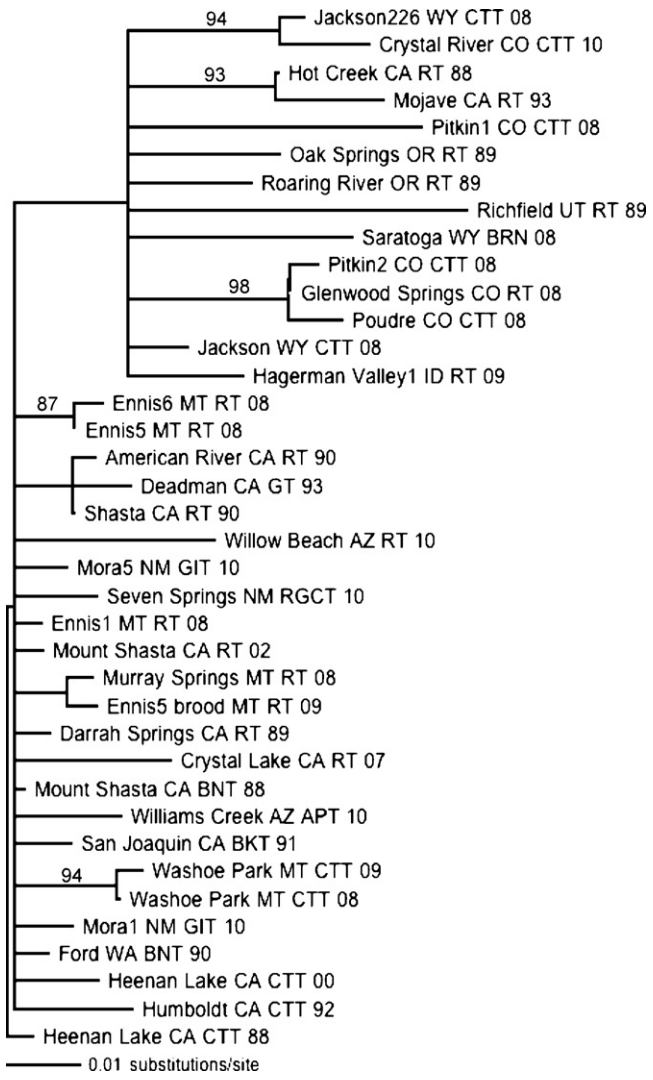

Fig. 5. Phylogenetic analysis of 38 sequence types of CTV from various years, geographic locations and trout species in the Western United States. The GenBank Accession Number for the sequence of the helicase domain corresponding to nucleotide positions 3178–3439 of the CTV genome are provided in Table 1.

the largest clade with isolates from CA, MT, WA, AZ and NM. A second clade suggested a weak association between certain isolates of CTV from CA and isolates from CO, UT, WY, ID and OR suggesting a separate epidemiological link.

3.5. Persistently infected cultures

Following approximately three weeks of incubation, CPE had progressed to involve most regions of the CHSE-214 cell monolayers, however many normal-appearing cells remained. After changing cell culture medium for several weeks, the monolayer began to regenerate, assuming a normal appearance (Fig. 6). Using aliquots of spent medium collected over an approximately 30-week period, RT-PCR assays showed the continued presence of CTV. The intensity of the bands seen in agarose gels remained relatively constant in spite of the extensive changes of culture medium, suggesting that relatively continuous synthesis of new virus was occurring.

4. Discussion

Our results indicate that the cutthroat trout virus is a candidate for inclusion in the family *Hepeviridae*, the first from an aquatic animal. Nucleotide sequence differences between CTV and hepeviruses from mammals or birds appeared to be sufficient to justify the creation of a novel genus, within the family that would include CTV as well as similar viruses isolated from fish or other aquatic animals in the future. We propose the genus name *Cutrovirus* to reflect the initial host, the cutthroat trout, with the 1988 Heenan Lake isolate as the type species. While CTV appears to be widespread among populations of spawning trout in the western United States, the sequence diversity among isolates from various locations was relatively low and the isolates did not appear to cluster with a phylogeographic signature seen with other hepeviruses (Lu et al., 2006; Okamoto, 2007; Aggarwal and Naik, 2009; Bilic et al., 2009; Marek et al., 2010) or with certain viruses affecting trout or salmon (Kurath et al., 2003; Snow et al., 2004). However, the reasons for the low level of diversity and apparent homogenization of sequence types are uncertain. Possible explanations include: (1) a limited period of active virus replication that occurs only briefly in salmonids prior to spawning, (2) the virus has spread to fish relatively recently and emerged without sufficient time for evolutionary processes to proceed, (3) the virus has another, yet undiscovered, reservoir that constrains evolutionary adaptation, or (4) the virus has been widely distributed by humans via the shipment of infected fish or eggs. At any rate, for newly discovered or emerging viruses of aquatic animals, there is limited understanding of the drivers of sequence diversity and the rate of evolutionary change that can be expected

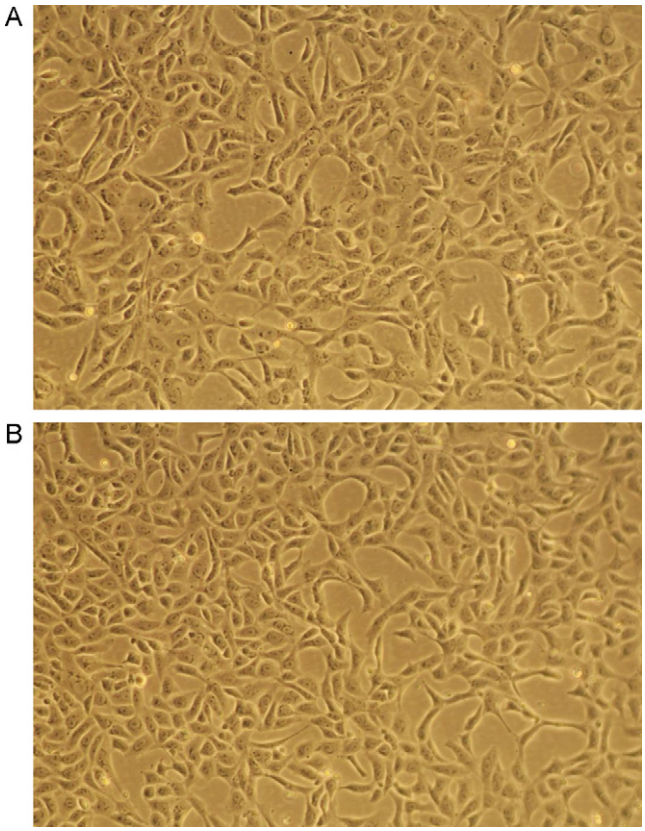

Fig. 6. Fish cell line persistently infected with the cutthroat trout virus (CTV). Cultures of the Chinook salmon embryo (CHSE-214) cell line were infected with the Heenan Lake isolate of CTV and allowed to go through a lytic cycle lasting several weeks during which nearly all cells were destroyed. The culture medium was replaced and surviving cells expanded to form a normal-appearing monolayer. The persistently infected culture could be propagated normally, yet continued to produce high levels of infectious virus. (A) Normal culture of CHSE-214 cells. (B) Persistently infected culture of CHSE-214 cells following more than seven months of serial passage.

within host-pathogen systems of poikilothermic vertebrates such as fish, reptiles and amphibians relative to that of their homeothermic counterparts (Domingo and Gomez, 2007).

To date, experimental infections of cutthroat trout, rainbow trout, brown trout, kokanee salmon (*O. nerka*), Chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutch*) with CTV have not been associated with mortality or microscopic pathology (Hedrick et al., 1991). This is in sharp contrast with *Hepatitis E virus* infections of humans that can lead to a broad range of outcomes including sub-acute, acute, chronic and fulminant forms of disease, often associated with hepatic lesions and mortality that is especially high in some regions of the world, particularly among women during later stages of pregnancy (Jaiswal et al., 2001; Jilani et al., 2007). The appearance of CTV in adult trout at spawning may be a useful model to study factors leading to induction of fulminant hepatitis E in women who have become pregnant or who are on oral contraception where high levels of steroid hormones are believed to exert immunosuppressive effects that exacerbate existing or acquired HEV infections (Jilani et al., 2007; Navaneethan et al., 2008; Lindemann et al., 2010). Analogous hormonal changes occur in rainbow trout during oogenesis and immediately prior to spawning (Nakamura et al., 2005; Lubzens et al., 2010). Because estrogens, progesterones or other corticosteroids and their analogs have been reported to suppress the immune response in fish (Watanuki et al., 2002; Pietsch et al., 2009; Thilagam et al., 2009), it would be interesting to determine if administration of these compounds to trout can exacerbate laboratory-acquired CTV infections or reactivate latent infections in younger fish.

Currently, it is uncertain how CTV is maintained in trout broodstock facilities. One possibility is that the virus is present in other reservoir species (analogous to zoonotic infections with HEV) or other trout life stages, and spreads to salmonid broodstocks as they approach spawning when they become more highly susceptible to infection as demonstrated for *Infectious hematopoietic necrosis virus* (Yamamoto et al., 1989). A second possibility is that CTV is maintained in a long-term carrier state as demonstrated for *Infectious pancreatic necrosis virus* (IPNV) where infections can persist for the life of the fish and the virus is efficiently transmitted horizontally to naïve animals as well as *intra ovum* from adult to progeny (Reno, 1999). In the case of IPNV, persistently infected CHSE-214 cell cultures could be readily initiated and used to model the carrier state in trout (Hedrick and Fryer, 1982). While a life-long carrier state and vertical transmission have not been experimentally demonstrated for CTV, long-term maintenance of the virus in populations and evidence for *intra ovum* transmission includes: finding similar CTV sequences in fish from the same hatchery over multiple years, finding similar sequences among fish at hatcheries linked by shipments of fish or eggs, and finding CTV infections in fish following surface disinfection of fertilized eggs and laboratory rearing on pathogen-free water supplies.

Results from our study show that CTV has been isolated over a 23-year span (1988–2010) from seven different trout species in 10 different watersheds of the western United States. While we are not aware of the isolation of CTV-like viruses from fish outside of North America, a virus was recovered from Atlantic salmon (*Salmo salar*) in New Brunswick, Canada suffering from a co-infection with the fish orthomyxovirus, *Infectious salmon anemia virus* (Kibenge et al., 2000). The virus, described as toga-like, had a partial nucleotide sequence (GenBank Number AF030878) that was 93% identical to the Heenan Lake 1988 strain of CTV, indicating that hepeviruses of fish are probably more widespread than presently known.

The apparent emergence of CTV in the last two decades may be a result of improved diagnostic and surveillance efforts or to an increase in the shipment of infected fish or eggs throughout the United States. While CTV grows well in a cell line (CHSE-214) that is used globally for detection of viruses infecting aquatic animals (OIE, 2009), CPE is relatively slow to develop and not especially dramatic. The RT-PCR assay described in this manuscript may be of assistance in confirming suspect cases or as a molecular assay to detect the virus in trout populations leading to better knowledge about the current distribution and epidemiology of CTV. Finally, the ability to propagate CTV to high titers both *in vitro* and *in vivo*, the availability of a susceptible animal model (albeit one in which pathology is absent), and the relative ease in establishment of what appeared to be persistently infected cell cultures may provide important research tools to advance our understanding of the molecular biology and immunology of hepeviruses and to serve as model systems for screening candidate vaccines or antiviral drugs.

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References

Aggarwal, R., Naik, S., 2009. Epidemiology of hepatitis E: current status. J. Gastroenterol. Hepatol. 24, 1484–1493.

Bilic, I., Jaskulska, B., Basic, A., Morrow, C.J., 2009. Sequence analysis and comparison of avian hepatitis E viruses from Australia and Europe indicate the existence of different genotypes. J. Gen. Virol. 90, 863–873.

Domingo, E., Gomez, J., 2007. Quasispecies and its impact on viral hepatitis. Virus. Res. 127, 131–150.

Emerson, S.U., Purcell, R.H., 2003. Hepatitis E virus. Rev. Med. Virol. 13, 145–154.

Fry, K.E., Tam, A.W., Smith, M.M., Kim, J.P., Luk, K.-C., Young, L.M., Piatak, M., Feldman, R.A., Reyes, G.R., et al., 1992. Hepatitis E virus (HEV): strain variation in the nonstructural gene region encoding consensus motifs for an RNA-dependent RNA polymerase and an ATP/GTP binding site. Virus Genes 6, 173–185.

Hedrick, R.P., Fryer, J.L., 1982. Persistent infections of salmonid cell lines with infectious pancreatic necrosis virus (IPNV): a model of the carrier state in trout. Fish Pathol. 16, 163–172.

Hedrick, R.P., Yun, S., Wingfield, W.H., 1991. A small RNA virus isolated from salmonid fishes in California, USA. Can. J. Fish. Aquat. Sci. 48, 99–104.

Hedrick, R.P., LaPatra, S.E., Yun, S., Lauda, K.A., Jones, G.R., Congleton, J.L., 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.

Huang, F.F., Sun, Z.F., Emerson, S.U., Purcell, R.H., Shivaprasad, H.L., Pierson, F.W., Toth, T.E., Meng, X.J., 2004. Determination and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV. J. Gen. Virol. 85, 1609–1618.

International Committee on Taxonomy of Viruses (ICTV), 2010. Virus taxonomy: 2009 Release. On-line at: http://www.ictvonline.org/virusTaxonomy.asp.

Jaiswal, S.P.B., Jain, A.K., Naik, G., Soni, N., Chitnis, D.S., 2001. Viral hepatitis during pregnancy. Int. J. Gynecol. Obstet. 72, 103–108.

Jilani, N., Das, B.C., Husain, S.A., Baweja, U.K., Chattopadhya, D., Gupta, R.K., Sardona, S., Kar, P., 2007. Hepatitis E virus infection and fulminant hepatic failure during pregnancy. J. Gastroenterol. Hepatol. 22, 676–682.

Johne, R., Heckel, G., Plenge-Bonig, A., Kindler, E., Maresch, C., Reetz, J., Schielke, A., Ulrich, R.G., 2010. Novel hepatitis E virus genotype in Norway rats, Germany. Emerg. Infect. Dis. 16, 1452–1455.

Kibenge, F.S.B., Whyte, S.K., Hammell, K.L., Rainnie, D., Kibenge, M.T., Martin, C.K., 2000. A dual infection of infectious salmon anaemia (ISA) virus and a togavirus-like virus in ISA of Atlantic salmon *Salmo salar* in New Brunswick, Canada. Dis. Aquat. Org. 42, 11–15.

Koonin, E.V., Gorbalenya, A.E., Purdy, M.A., Rozanov, M.N., Reyes, G.R., Bradley, D.W., 1992. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. Proc. Natl. Acad. Sci. U. S. A. 89, 8259–8263.

Kurath, G., Garver, K.A., Troyer, R.M., Emmenegger, E.J., Einer-Jensen, K., Anderson, E.D., 2003. Phylogeography of infectious haematopoietic necrosis virus in North America. J. Gen. Virol. 84, 803–814.

Lindemann, M.L.M., Morales, J.G., Fernandez-Barredo, S., Dominguez, M.R., de la Hoz, F.G., Halfon, P., Gracia, M.T.P., 2010. Case report: fulminant hepatitis E in a woman taking oral contraceptive medication. Am. J. Trop. Med. Hyg. 82, 12–15.

Lu, L., Li, C., Hagedorn, C.H., 2006. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. Rev. Med. Virol. 16, 5–36.

Lubzens, E., Young, G., Bobe, J., Cerda, J., 2010. Oogenesis in teleosts: how fish eggs are formed. Gen. Comp. Endocrinol. 165, 367–389.

Marek, A., Bilic, I., Prokofieva, I., Hess, M., 2010. Phylogenetic analysis of avian hepatitis E virus samples from European and Australian chicken flocks supports the existence of a different genus within the Hepeviridae comprising at least three different genotypes. Vet. Microbiol. 145, 54–61.

Meng, X.J., 2010. Recent advances in hepatitis E virus. J. Viral Hepatitis 17, 153–161.

Mushahwar, I.K., 2008. Hepatitis E virus: molecular virology, clinical features, diagnosis, transmission, epidemiology, and prevention. J. Med. Virol. 80, 646–658.

Nakamura, I., Evans, J.C., Kusabe, M., Nagahama, Y., Young, G., 2005. Changes in steroidogenic enzyme and steroidogenic acute regulatory protein messenger RNAs in ovarian follicles during ovarian development of rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 144, 224–231.

Navaneethan, U., Al Mohajer, M., Shata, M.T., 2008. Hepatitis E and pregnancy: understanding the pathogenesis. Liver Int. 28, 1190–1199.

Office International des Epizooties (OIE), 2009. Manual of Diagnostic Tests for Aquatic Animals, sixth ed. World Organization for Animal Health, Paris.

Okamoto, H., 2007. Genetic variability and evolution of hepatitis E virus. Virus Res. 127, 216–228.

Panda, S.K., Thakral, D., Rehman, S., 2007. Hepatitis E virus. Rev. Med. Virol. 17, 151–180.

Pietsch, C., Neumann, N., Knopf, K., Wuertz, S., Kloas, W., 2009. Progestogens cause immunosuppression of stimulated carp (*Cyprinus carpio* L.) leukocytes in vitro. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 150, 16–24.

Purcell, R.H., Emerson, S.U., 2008. Hepatitis E: an emerging awareness of an old disease. J. Hepatol. 48, 494–503.

Reno, P.W., 1999. Infectious pancreatic necrosis and associated aquatic birnaviruses. In: Woo, P.T.K., Bruno, D.W. (Eds.), Fish Diseases and Disorders Volume 3 Viral, Bacterial and Fungal Infections. CABI, New York.

Rozanov, M.N., Koonin, E.V., Gorbalenya, A.E., 1992. Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses. J. Gen. Virol. 73, 2129–2134.

Snow, M., Bain, N., Black, J., Taupin, V., Cunningham, C.O., King, J.A., Skall, H.F., Raynard, R.S., 2004. Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). Dis. Aquat. Org. 61, 11–21.

Swofford, D.L., 1998. PAUP* v 4: Phylogenetic Analysis Using Parsimony (*and Other Methods). Sinauer Associates, Sunderland, MA, USA.

Tam, A.W., Smith, M.M., Guerra, M.E., Huang, C.-C., Bradley, D.W., Fry, K.E., Reyes, G.R., 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185, 120–131.

Thilagam, H., Gopalakrishnan, S., Bo, J., Wang, K.-J., 2009. Effect of 17ss-estradiol on the immunocompetence of Japanese sea bass (*Lateolabrax japonicas*). Environ. Toxicol. Chem. 28, 1722–1731.

Vasickova, P., Psikal, I., Kralik, P., Widen, F., Hubalek, Z., Pavlik, I., 2007. Hepatitis E virus: a review. Vet. Med. 52, 365–384.

Watanuki, H., Yamaguchi, T., Sakai, M., 2002. Suppression in function of phagocytic cells in common carp *Cyprinus carpio* L. injected with estradiol, progesterone or 11-ketotestosterone. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 132, 407–413.

Yamamoto, T., Arakawa, C.K., Batts, W.N., Winton, J.R., 1989. Comparison of infectious hematopoietic necrosis in natural and experimental infections of spawning salmonids by infectivity and immunohistochemistry. In: Ahne, W., Kurstak, E. (Eds.), Viruses of Lower Vertebrates. Springer-Verlag, Heidelburg, pp. 411–429.