Use of cell lines and primary cultures to explore the capacity of rainbow trout to be a host for frog virus 3 (FV3)

P. H. Pham[1] · Y. J. Huang[1] · D. D. Mosser[2] · N. C. Bols[1]

Abstract The capacity of rainbow trout, *Oncorhynchus mykiss*, to be a host for frog virus 3 (FV3) was evaluated at the cellular level. Cell cultures from this species were tested for their ability to express FV3 major capsid protein (MCP) gene, to develop cytopathic effect (CPE), and to produce FV3. After FV3 addition, MCP transcripts were detected in six of six cell lines and in primary macrophage cultures. CPE developed in all cell culture systems, except primary lymphocytes. For the macrophage cell line, RTS11, and primary macrophages, cell death was by apoptosis because DNA laddering and Annexin staining were detected. By contrast, markers of apoptosis did not accompany CPE in three epithelial cell lines from the gill (RTgill-W1), intestine (RTgut-GC), and liver (RTL-W1) and in two fibroblast cell lines from gonads (RTG-2) and skin (RTHDF). Therefore, FV3 was able to enter and begin replicating in several cell types. Yet, FV3 was produced in only two cell lines, RTG-2 and RTL-W1, and only modestly. Overall, these results suggest that if tissue accessibility were possible, FV3 would have the capacity to induce injury, but the ability to replicate would be limited, likely making rainbow trout a poor host for FV3.

Keywords Frog virus 3 (FV3) · Ranavirus · Apoptosis · Rainbow trout · Macrophages · Lymphocytes · Head kidney · Liver · Gut · Gills · Skin · Gonads

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Introduction

Frog virus 3 (FV3) is the type species of the genus Ranavirus within the family Iridoviridae (Chinchar *et al.* 2011; Chinchar *et al.* 2014), and has been linked to die-offs of frogs and salamanders (Miller *et al.* 2011). Viruses of the Iridoviridae are icosahedral and enveloped, with single linear double-stranded DNA genomes that encode for 100–140 proteins. FV3 and FV3-like viruses infect and cause disease in different classes of ectothermic vertebrates (Brenes *et al.* 2014a). Less is known about how FV3 acts in fish, but fish species could be incidental hosts, reservoir hosts, or amplifying hosts for viruses infecting other vertebrate classes (Bollinger *et al.* 1999; Ashford 2003; Brenes *et al.* 2014a, b). To date, FV3-like ranaviruses have been isolated from only a few warm water fish species, such as the pallid sturgeon (Brenes *et al.* 2014a; Waltzek *et al.* 2014). When fish are experimentally infected, FV3 and FV3-like ranaviruses appear to cause low or no mortality (Jensen *et al.* 2009, 2011a, b; Gobbo *et al.* 2010; Picco *et al.* 2010; Brenes *et al.* 2014a; Leimbach *et al.* 2014).

Whether FV3 can interact with rainbow trout in ways that would allow this species to be a FV3 host is unclear, but is important to know for several reasons. Rainbow trout is a highly valued species in recreational fishing, aquaculture, and research, especially in environmental toxicology. FV3 appears to be ubiquitous in the temperate freshwater environment of rainbow trout and impacts rainbow trout prey, tadpoles and frogs (Miller *et al.* 2011; Nazir *et al.* 2012). These food items would contain the virus in subclinical infections and in carcasses during mass mortality events (Miller *et al.* 2011). When 50 rainbow trout fry were inoculated with FV3, the virus could be isolated 7 d later from only one fish, and no pathological changes were observed (Ariel *et al.* 2010).

The interactions between viruses and cells of multicellular animals determine the host species for a virus, the kinds of

pathological effects that the virus can cause in animals, or the possible benefits that can accrue to animals from association with a virus. The latter is a newer concept, which is termed mutualistic symbiosis and poorly understood (Virgin 2014). To be a host, the cells of a species must allow a virus to enter, replicate, and exit (Webby et al. 2004). This leads to virus production, but this cellular capability alone might not be enough to define a host because organismal and populations level barriers might prevent cells from being exposed to the virus. Virus production is often accompanied by cell death, usually apoptosis, but other cell death mechanisms may be involved (Roulston et al. 1999; Mocarski et al. 2014). Cell death leads to pathological changes in animals but also under some circumstances prevent the virus from being produced in animals (Upton and Chan 2014).

Cultures of cell lines and/or primary cells are arguably one of the most important approaches for investigating virus/animal cell interactions, and for FV3 and fish, have been used most intensively with two fathead minnow epithelial cell lines, FHM and EPC. FV3 production in FHM was optimal at 31–32°C and could occur at temperatures as low as 12°C, but not at 10°C (Gravell and Granoff 1970). FV3 caused cell death or cytopathic effect (CPE) in FHM cultures, and when examined in cultures at 24°C, cell death was by apoptosis (Chinchar et al. 2003). EPC also supported FV3 production at 22°C (Ariel et al. 2009; Holopainen et al. 2011; 2012) and responded to FV3 with an increased expression of interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) (Holopainen et al. 2012). Another warm water fish cell line, bluegill fry 2 (BF-2), also supported virus replication at 20 and 24°C, but the rainbow trout gonadal fibroblast cell line, RTG-2, did so poorly (Ariel et al. 2009). These studies have been invaluable for identifying how to detect and propagate the virus in vitro.

Recently, in mammalian virology, batteries of cell lines focused around themes, such as an animal group, species, or tissue, and have been screened for their responses to a particular virus to understand the host and tissue tropism of the virus (Chan et al. 2013). Therefore, in this study, a panel of cell lines from different rainbow trout tissues and organs as well as in primary leucocyte cultures was screened for their responses to FV3. This was done at 20–22°C because rainbow trout cells in culture grow best at these temperatures (Bols et al. 1992) and because although slightly above the optimal growth temperature for rainbow trout, this species can feed at up to 22°C (Rebel et al. 2013). For all cell lines, FV3 entered and caused cell death but only the macrophage cell line (RTS11) died by apoptosis and the virus was produced in just RTG-2 and a liver epithelial cell line, RTL-W1, and only modestly. Therefore, rainbow trout is likely to be a poor host for FV3 but could be damaged by FV3.

Materials and Methods

FV3 propagation and quantification. Frog virus 3 (FV3) was obtained from Dr C. Brunetti at Trent University, Peterborough, Ontario (Eaton et al. 2008). The virus was propagated and titrated on EPC, an epithelial-like cell line from fathead minnow (Eaton et al. 2008; Winton et al. 2010). Propagation was done on EPC cells in 75-cm^2 flasks (BioLite, Thermo Scientific, Mississauga, Canada) at 26°C in L15 medium with 2% fetal bovine serum (FBS) (PAA Laboratories, VWR International, Mississauga, Canada). Seven days post infection (pi), the entire content of the flasks was collected and centrifuged at 4000×g for 5 min to pellet cellular debris. The supernatant was collected, syringe filtered through a 0.2-μm filter (Pall Corporation, Thermo Scientific, Mississauga, Canada), separated into aliquots, and stored at −80°C. Viral titre was determined using the Karber method of tissue culture infectious dose (TCID$_{50}$/mL) (Karber et al. 1931).

Cell lines. Immune and non-immune cell lines from fish were used. The immune cell lines were RTS11 from rainbow trout. RTS11 arose from a spleen long-term hematopoietic culture, grows loosely adherent or in suspension, and has properties that lead them to be called monocyte/macrophage-like (Ganassin and Bols 1998). Besides EPC, described in the previous section, the non-immune cell lines were from rainbow trout and grew adhered to the plastic surface of culture vessels. Three of these were epithelial-like and were RTL-W1 from liver (Lee et al. 1993), RTgut-GC from the gastrointestinal tract (Kawano et al. 2011), and RTgill-W1 from the gill (Bols et al. 1994). The two fibroblast-like cell lines were RTG-2 from gonad (Wolf and Quimby 1962) and RTHDF from skin (Ossum et al. 2004).

The foundation of the growth media was Leibovitz's L-15 (Hyclone, Thermo Scientific, Mississauga, Canada). This was supplemented with FBS and 1% penicillin-streptomycin (PS) (Hyclone, Thermo Scientific). RTgill-W1, RTL-W1, RTgut-GC, and RTG-2 were grown in L-15 with 10% FBS. For RTHDF and RTS11, the L15 was supplemented with 15% FBS. Cell lines were subcultivated at a ratio of 1:2 once every 1 to 2 wk.

Infection of cell line cultures with FV3. For each cell line, infection with FV3 (final titre of approximately 10^7 TCID$_{50}$/mL) or mock infection with control medium was performed with approximately 10^6 cells/well in triplicate wells of a 6-well plate. The initial infection of cell lines was performed differently for adherent cells than those that grew in suspension. Cells that grew in suspension were RTS11. Adherent cells were detached with trypsin-versene (Lonza, Thermo Scientific, Mississauga, Canada) and seeded into 6-well plates

in their growth medium. The plates were incubated at room temperature overnight to allow attachment of cells. The following day, old medium was removed and each well was washed 1× with Dulbecco`s phosphate buffered saline (DPBS) (Lonza, Thermo Scientific); triplicate wells of each 6-well plate were then infected with FV3 in 2 mL of L15 containing 2% FBS (2% FBS/L15) or in the case of control cells, mock infected with 2 mL of 2% FBS/L15 containing no virus. For cells that grew in suspension, the cells were collected from their growth flasks, pelleted at 440×g for 5 min and washed 1× with DPBS. Cells were then either infected with FV3 in 2 mL of 2% FBS/L15 or mock infected with 2 mL of 2% FBS/L15 before being seeded into triplicate wells of a 6-well plate. Plates were incubated at 20°C for either observation of cytopathic effect (CPE), or further downstream experiments such as resazurin cell viability assay, apoptosis assays, or RT-PCR.

Preparation of rainbow trout immune cell suspensions. Rainbow trout with average weight of approximately 250 g (Lyndon Hatchery, New Dundee, Canada) were anesthetized in approximately 1 mL/L of 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO). Peripheral blood was taken from the caudal vein with a 22 G needle coated with heparin (Sigma-Aldrich). The blood was layered over Histopaque-1077 (Sigma-Aldrich) and centrifuged at 400×g for 30 min. The cells at the serum/Histopague-1077 interface were collected and transferred to a second Histopaque-1077 layer for a second 30-min centrifugation at 400×g. The cells at the interface were collected again, washed 2× with DPBS, and resuspended in L15 with 10% FBS for subsequent experiments. Head kidney tissues were collected and suspended in L15 with 10% FBS. The tissue was vortexed vigorously to dissociate cells and passed through a 100 μm cell strainer (Thermo Scientific). The medium containing the cells was subsequently added to a layer of Histopaque-1077 and centrifuged at 400×g for 30 min. The cells at the interface were collected, washed 2× with DPBS, and resuspended in L15 with 10% FBS for subsequent initiation of primary cell cultures and experiments.

Preparation and infection of rainbow trout primary immune cell culture with FV3. From the head kidney and peripheral blood leucocyte (PBL) suspensions, cultures of adherent and non-adherent cells were established. The suspended cells were seeded into wells of 6-well plates at approximately 5.0×10^6 cells/mL. The plates were incubated overnight at 14°C. The next day, some cells had attached to the plastic surface while the remainder was in suspension in the medium. This medium was added to new 6-well plates to give cultures of immune cells in suspension. Although a very small fraction of these cells are red blood cells or other cells that would subsequently attach to the culture surface over the next 72 h, most cells

remained in suspension and are considered lymphocytes. The initial 6-well plates now had just adherent cells, after the removal of the medium with the floating cells, and fresh medium was added to maintain these cells. These adherent cells appeared macrophage-like, although a few epithelial-like cells were also present. Adherent and non-adherent cultures were infected with FV3 as described for the cell lines, incubated at 20°C, and evaluated for cell viability and gene expression as outlined below.

Determining an effect of FV3 on cell viability. After cultures of the cell lines and of the rainbow trout immune cells had been infected with FV3 as described, respectively, in Sections 2.3 and 2.5; the loss of cell viability was evaluated in several ways. One was to monitor the phase contrast appearance of cultures. For adherent cultures, a loss of viability was indicated by disruption of the monolayer and the accumulation of cellular debris in the medium. For suspension cultures, a loss of viability was shown by the appearance of phase dark rather than phase bright cells and the appearance of cellular debris. As well, the oxidation-reduction indicator dye, resazurin, was used to evaluate cell viability in suspension cultures (Dayeh *et al.* 2013). Upon reduction by living cells resazurin becomes fluorescent, which can be measured as relative fluorescent units (RFUs). A decline in the ability of cells to reduce resazurin is seen as a decline in RFUs and can be interpreted as an impairment of cellular metabolism (Rampersad *et al.* 2012; Dayeh *et al.* 2013). A commercial preparation of resazurin called alamarBlue was used (Life Technologies Inc, Burlington, Canada). At specified time points, 2 mL of 10% alamarBlue solution diluted in DPBS was added to each well containing 2 mL of cells and virus, making a final alamarBlue concentration of 5%. The plates were incubated in the dark at room temperature for 3 h before being measured in a CytoFluor fluorescent plate reader (PerSeptive Biosystems). The output of the measurement is in RFU, and percent metabolic impairment was determined by dividing the RFU of FV3 exposed wells with RFU of control wells and multiplying by 100.

Evaluating the ability of FV3 to induce hallmarks of apoptosis. The two methods used to determine apoptosis in the cell lines were DNA laddering by agarose gel electrophoresis and Annexin V-PE staining for externalization of phosphatidylserine across the cell membrane. Cell lines were either infected with FV3 or mock infected as described in Section 2.3. For the DNA laddering assay, adherent cells were collected using trypsin-versene while non-adherent cells were collected directly without any detachment solution at specific time points. Cells were pelleted at 1000×g for 5 min and total genomic DNA was extracted using the E.Z.N.A Tissue DNA Kit (Omega bio-tek, VWR International, Mississauga, Canada). The collected DNA samples were electrophoresed

on a 2% agarose gel for up to 2 h and subsequently imaged. Detection of phosphatidylserine was done using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, Mississauga, Canada). Quantification of apoptotic cells was conducted using a Guava EasyCyte Mini and associated software (Millipore Corporation, Etobicoke, Canada).

Evaluating the ability of cell lines and primary cultures to support FV3 gene expression. Cells and total primary culture head kidney leucocytes were infected as described in Sections 2.3 and 2.5, respectively. At specific time points, adherent cells were collected using trypsin-versene and non-adherent cells collected directly without any detachment solution. Cells were centrifuged at 440×*g* for 5 min and washed with DPBS; centrifugation and washing was repeated for a total of three times. Total RNA was extracted from each cell sample using the E.Z.N.A Total RNA Kit I (Omega bio-tek, VWR International), and first-strand synthesis was performed using either the RevertAid H Minus Reverse Transcriptase Kit (Fermentas, Thermo Scientific, Mississauga, Canada) or Promega ImProm-II Reverse Transcription System (Promega, Thermo Scientific, Mississauga, ON). Gene amplification was performed using recombinant Taq DNA polymerase (Fermentas, Thermo Scientific) with primers for β-actin (forward primer 5′-ATCGTGGGGCGCCCCAGGCACC-3′ and reverse primer 5′-CTCCTTAATGTCACGCACGATT TC-3′) (Brubacher *et al.* 2000) and major capsid protein (MCP) gene (forward primer 5′-GACTTGGCCACTTATG AC-3′ and reverse primer 5′-GTCTCTGGAGAAGAAGAA-3′) (Mao *et al.* 1996). The cycling conditions were as follows: initial denaturation at 95°C for 3 min; 40 amplification cycles of 94°C for 1 min, primer annealing at 55°C for 45 s, and elongation for 45 s; and a final extension at 72°C for 7 min. PCR products were electrophoresed on a 1% agarose gel and subsequently imaged. RT-PCR was performed on two independent experiments for each cell line.

Evaluating the ability of rainbow trout cell lines to support FV3 production. Cell lines were infected with FV3 as described in Section 2.3, and incubated for 2 h before the old medium was removed. The cells were then washed 3× with 2 mL of D-PBS before 2 mL of fresh 2% FBS/L15 was added. For RTS11, each washing step required centrifugation at 440×*g* for 5 min to pellet the cells. For the day zero time point, 100 μL of supernatant was collected immediately after the fresh medium was added to the cells, and stored at −80°C for subsequent titration. After collection, 100 μL of new medium was added back into the wells and the cells were incubated at 20°C for up to 10 d. Collection of samples for the other time points was done as described for day zero. Viral titre was determined using the Karber method of tissue culture infectious dose (TCID50/mL) (Karber *et al.* 1931).

Results

Major capsid protein gene expression as a marker of FV3 entry into rainbow trout cells. In order to determine whether FV3 can enter and initiate replication in rainbow trout cells, primary cell cultures and cell lines were examined by RT-PCR for the expression of transcripts for the FV3 major capsid protein, which is known to be expressed late in the FV3 replication cycle (Chinchar *et al.* 2011). The positive control was the fathead minnow cell line, EPC, which is used to routinely propagate the virus (Fig. 1). MPC transcripts were seen at 6 and 12 h post infection (pi) in primary cultures of rainbow trout head kidney cell leukocytes (HKL) (Fig. 1). At 24 and 48 h after the introduction of FV3, MPC transcripts were also detected in cultures of three epithelial cell lines, RTgut-GC, RTL-W1, and RTgill-W1, of two fibroblast cell lines, RTG-2 and RTHDF, and one monocyte macrophage cell line, RTS11. RTgut-GC, RTL-W1, and RTgill-W1 were derived, respectively, from the rainbow trout intestine, liver, and gill. RTG-2 originated from gonads and RTHDF from skin. RTS11 developed from a long-term spleen hemopoietic culture. Therefore, FV3 appears to be able to enter and start replicating in different cell types from a range of rainbow trout organs.

FV3 cytopathic effect in primary macrophage and RTS11 cultures. FV3 caused cytopathic effect (CPE) in cultures of the macrophage-like cells that adhered from suspensions of peripheral blood leucocyte (PBL) and the HKL cells but not the non-adherent lymphocyte-like cells. Phase contrast observations revealed that, in adherent cell cultures 24 h pi with FV3, a significant proportion of macrophages had been lost from the surface and replaced by cellular debris in suspension or loosely attached to the surface (Fig. 2*a, b*). By contrast, cultures of non-adherent cells with FV3 continued to look similar to control cultures not exposed to FV3, with floating phase bright cells and little cellular debris. FV3 also had a very different impact on the capacity of the two types of cultures to reduce alamarBlue. For floating lymphocyte-like cells at 24 h pi, those from PBL (PBL-F) showed no change in the ability to reduce alamarBlue (Fig. 2*c*), and those from the HKL (HKL-F) showed only a slight drop, with capacity at 80 to 90% of the control level (Fig. 2*c*). By contrast, the reduction of alamarBlue by adherent macrophage-like cultures from PBL (PBL-A) and HKL (HKL-A) dropped significantly, with, respectively, 40 to 50% and 10 to 20% of control level at 24 h pi with FV3 (Fig. 2*c*).

FV3 was also destructive to RTS11 cultures. Over 24 h, cultures with FV3 were observed by phase contrast microscopy and seen to accumulate cellular debris and phase dark cells (Fig. 2*d*). Also, the ability to reduce the indicator dye, alamarBlue, dropped dramatically. Relative to control cultures, FV3-infected cultures reduced alamarBlue at 50 to
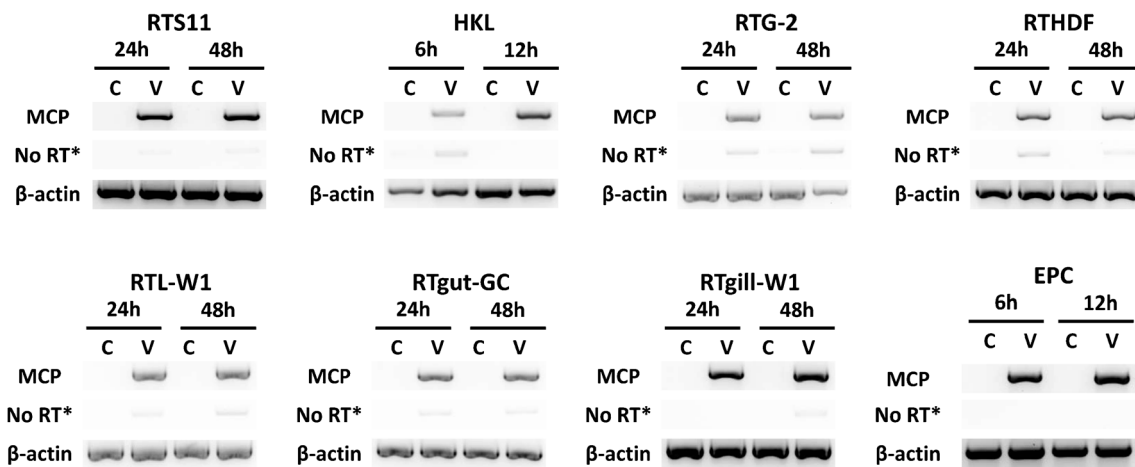
Figure 1. Expression of FV3 transcripts in cell lines and primary cultures. One transcript, the major capsid protein (*MCP*) expressed at the late stage of the FV3 replication cycle were probed in the rainbow trout cell lines, primary head kidney leucocytes (*HKL*) culture, and the positive control EPC cell line. MCP transcripts were detected in all rainbow trout cell lines and primary HKL culture, and the EPC cell line. To limit viral genomic DNA contamination, RNA preparations were DNase treated and one set of samples from the same RNA preparation underwent the reverse transcription reaction without reverse transcriptase (*RT*) in the mixture.

60% of the level of control cultures at 24 h pi, and at 72 h pi, this had dropped to 20 to 30% (Fig. 2*c*). Therefore, FV3 appeared to kill adherent macrophage-like cells from primary culture and the monocyte/macrophage cell line, RTS11.

The underlying mechanism behind the CPE in immune cells was investigated, beginning with RTS11. Two assays were used to determine whether death was by apoptosis: DNA laddering and Annexin V-PE staining. DNA fragmentation, in the form of DNA laddering on agarose gel electrophoresis, was observed in RTS11 infected with FV3. For RTS11, DNA laddering was clearly seen at approximately 24 h pi (Fig. 3*a*); the 48 h pi time point was not shown because most of the cells in the culture infected with FV3 were dead, which left an insufficient amount of genomic DNA for collection. Annexin V-PE staining was done 24 h after RTS11 cultures had been either FV3-infected or mock-infected (controls). RTS11 cultures infected with FV3 showed an average of approximately 44.80±1.59% of annexin V positive cells at 24 h pi while base level in control showed an average of 11.13 ±0.29% (Fig. 3*b*).

For macrophage-like cells from the primary culture head kidney leucocyte (HKL-A), the FV3-infected cultures showed an average of 36.13±0.55% annexin V positive cells while the control cultures showed an average of 13.17±2.22% at 1 d post infection (Fig. 3*b*). In contrast, the lymphocyte-like cells from primary culture head kidney leucocyte (HKL-F) showed a high base level of annexin positive cells in control cultures at an average of 35.97± 1.45%, but the levels did not significantly increase when the cultures were infected with FV3, remaining at an average of 41.27±3.41% at 1 d post infection (Fig. 3*b*). Therefore, FV3 caused the death of RTS11 and of macrophage-like cell in primary culture head kidney leucocytes by apoptosis.

FV3 CPE in epithelial and fibroblast cell lines. As judged by phase contrast microscopy, the addition of FV3 to monolayer cultures of the epithelial and fibroblast cell lines elicited damage to the monolayers when compared to mock-infected control cultures. Within the first 3 d, many cells had rounded and begun to lift off the plastic growth surface, while concurrently debris appeared in cultures. These changes are illustrated for RTG-2 (Fig. 4*a*) and RTHDF after 24 h pi (Fig. 4*b*); RTL-W1 (Fig. 4*c*), RTgut-GC (Fig. 4*d*), and EPC after 48 h pi (Fig. 4*e*); and RTgill-W1 after 72 h pi (Fig. 4*f*).

FV3 failed to induce markers of apoptosis in cultures of the fibroblast and epithelial cell lines, including EPC. DNA laddering was not seen in these cultures (Fig. 3*a*) and the level of annexin positive cells did not vary significantly between control and FV3-infected cultures at 1 d post infection (Fig. 3*b*).

FV3 production by rainbow trout cell lines. Cultures of six rainbow trout cell lines were evaluated for their ability to support an increase in FV3 titre but only two cell lines appeared to have this ability (Fig. 5). The starting point in each case was the titre at time zero, which was the titre after the initial virus-containing medium had been removed and the culture rinsed. The results were compared to the fathead minnow cell line, EPC, which was used to routinely propagate the virus. EPC produced approximately $10^{9.70}$ TCID50/mL by day 10 pi (Fig. 5). By contrast, the best rainbow trout cell line, RTG-2, produced approximately $10^{7.45}$ TCID50/mL by day 10 pi compared to day zero titre of $10^{4.53}$ TCID50/mL (Fig. 5). RTL-W1 culture also supported an increase in titre over 10 d. The average titre of FV3 in RTL-W1 reached $10^{5.70}$ TCID50/ mL by day 10 pi, which was more than 1 log higher than the day zero titre ($10^{4.12}$ TCID50/mL; Fig. 5). However, FV3 titres in cultures of the epithelial cell lines, RTgill-W1 and
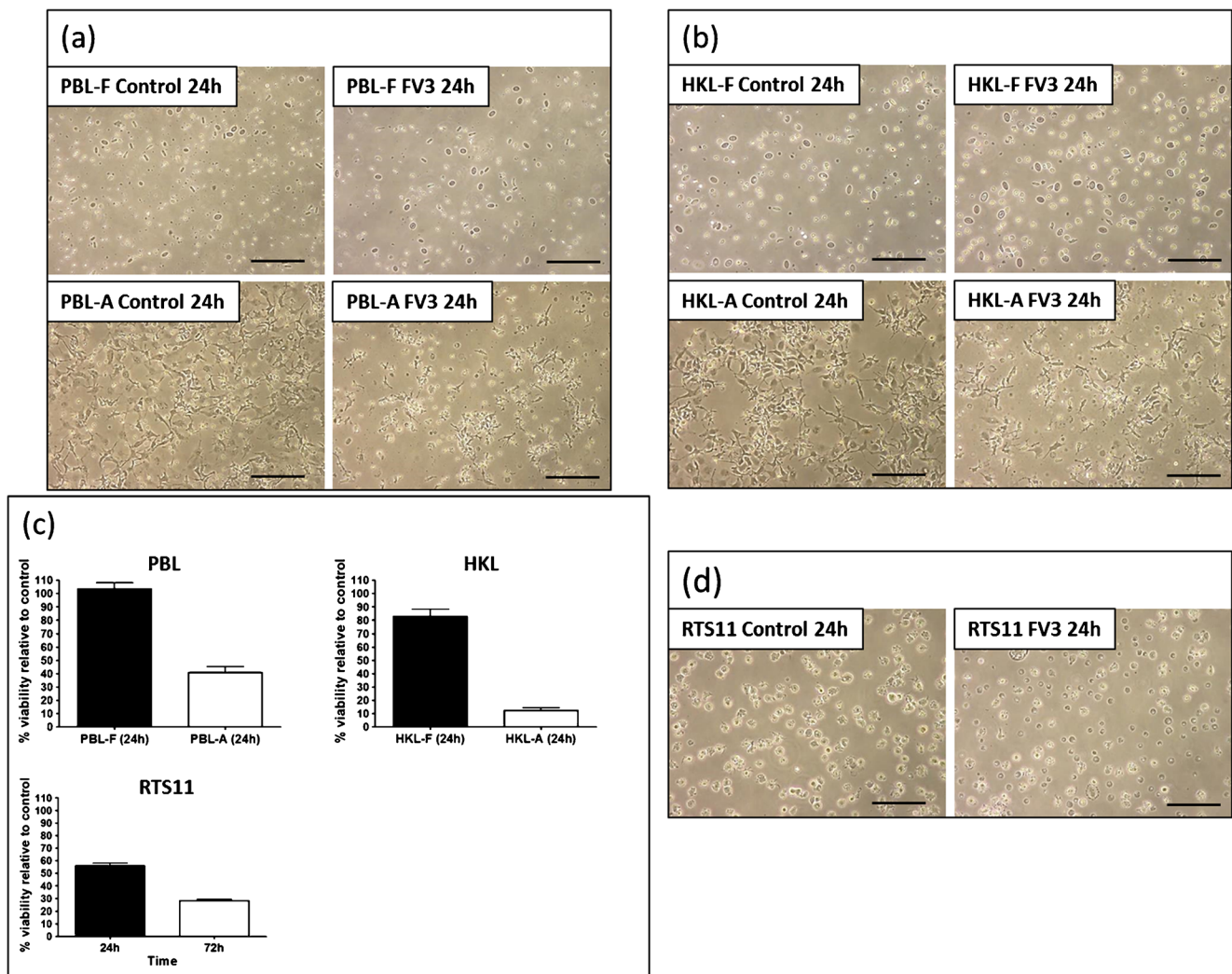
Figure 2. Observations of cytopathic effect (*CPE*) and metabolic activity of rainbow trout primary leucocyte cultures infected with FV3. Peripheral blood leucocytes (*PBL*) were separated into two fractions to produce the floating lymphocyte-like fraction (*PBL-F*) and the adherent macrophage-like fraction (*PBL-A*); a similar separation was performed for head kidney leucocytes (*HKL*) to produce floating lymphocyte-like fraction (*HKL-F*) and adherent macrophage-like fraction (*HKL-A*). (*a*) PBL fractions, (*b*) HKL fractions, and (*d*) RTS11 cultures were infected with either FV3 or control medium at 20°C; these cultures were monitored visually for CPE or (*c*) measured for reduction in metabolic activity by the alamarBlue cell viability assay. The percentage viability of FV3 infected cultures were determined relative to mock-infected control cells. *Data points* represent $N=3\pm SD$.

RTgut-GC, the fibroblast cell line, RTHDF, and the monocyte/macrophage cell line, RTS11, were all less than 1 log from their day zero titre by day 10 pi, indicating that these cell lines did not support a significant amount of FV3 replication (Fig. 5). Therefore, only the fibroblastic RTG-2 and epithelial RTL-W1 appeared to support production of FV3, and relative to EPC, they did so poorly (Fig. 5).

Discussion

Frog virus 3 (FV3) was able to enter a range of rainbow trout cell types and elicit either apoptotic or non-apoptotic cell death, suggesting that the virus has the potential to cause

tissue injury in this fish species. Yet FV3 was produced poorly and only by two of six rainbow trout cell lines. The very modest ability of rainbow trout cells to support FV3 replication would be a strong barrier at the cellular level to rainbow trout being a FV3 host.

FV3 entered and initiated replication in all rainbow trout cell cultures systems. Based on the detection of major capsid protein (MCP) transcripts by RT-PCR, a wide range of rainbow trout cells were susceptible to FV3 entry and supported the initiation and development of viral replication to the late events of the FV3 life cycle. MCP transcripts were seen after the addition of FV3 to primary leukocyte cultures and to cultures of three epithelial cell lines, RTL-W1 from the liver (Lee

Figure 3. Detection of apoptosis in cell lines. (*a*) Induction of DNA fragmentation in cell lines infected with either FV3 or control medium. DNA laddering was observed only for RTS11. The rest of the cell lines did not show DNA fragmentation when infected with FV3. (*b*) Percentage of annexin positive cells quantified in 1 d post FV3 or mock (control medium) infected cultures. Only RTS11 and macrophage-like cultures from primary culture head kidney leucocytes (*HKL-A*) showed increased levels of annexin positive cells in response to FV3 infection. *Data points* represents $N=3\pm SD$ and *** indicates a significance level of $p<0.001$.
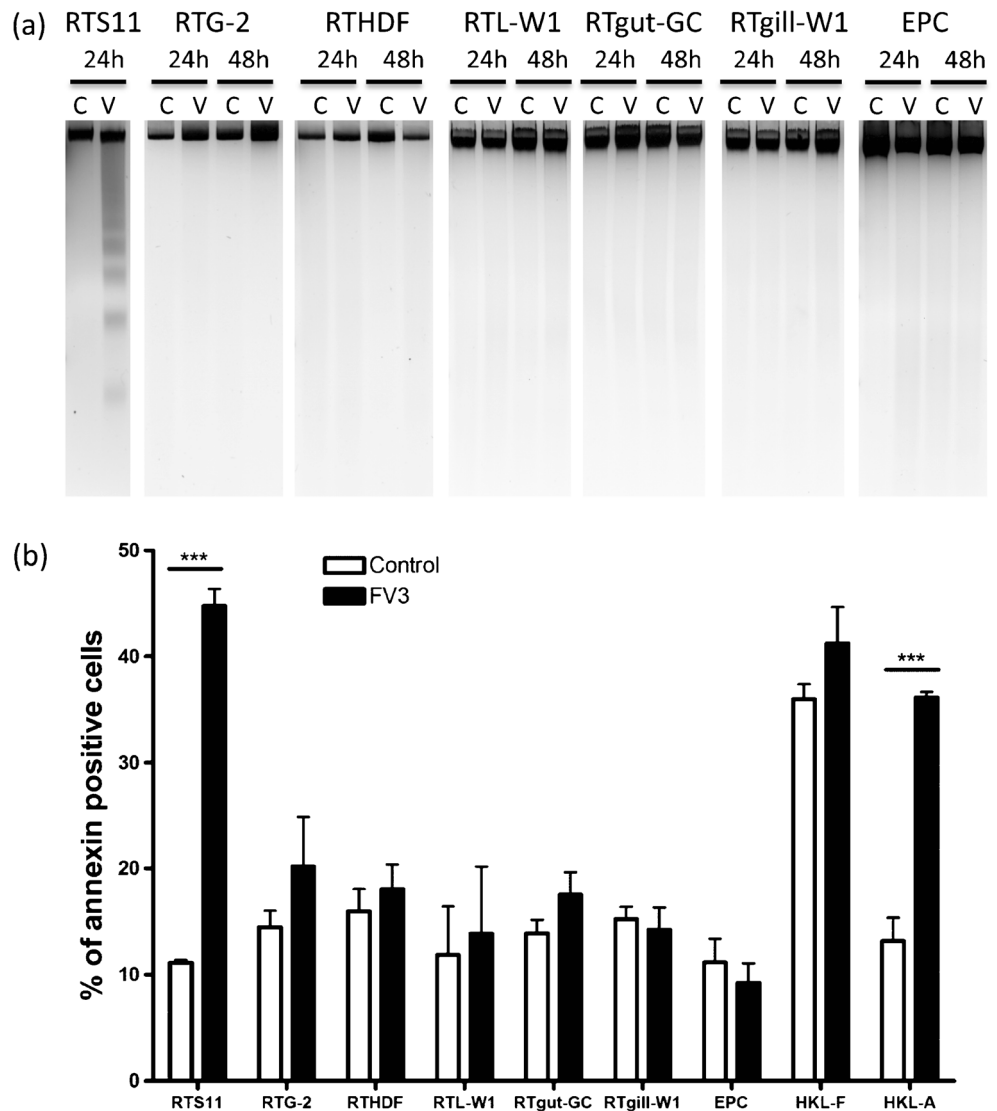
et al. 1993), RTgill-W1 from the gill (Bols *et al.* 1994), and RTgut-GC from the gut (Kawano *et al.* 2011), of two fibroblast cell lines, RTG-2 (Wolf and Quimby 1962) from gonads and RTHDF from skin (Ossum *et al.* 2004), and a monocyte/macrophage cell line RTS11 from the spleen (Ganassin and Bols 1998). FV3 entry into a wide range of cell types might be expected because in most viral preparations, two forms are present, non-enveloped (naked) particles and enveloped particles (Chinchar *et al.* 2011). The naked particles are thought to enter by fusion with the plasma membrane; enveloped particles, by receptor-mediated endocytosis (Chinchar *et al.* 2011), although evidence for an atypical, pH-dependent, caveola-mediated endocytic pathway has been presented for tiger frog virus (TFV) entry into human liver cells (Guo *et al.* 2011). As fusion and receptor-mediated endocytosis are functions common to vertebrate cells, one or both would likely be available in nearly all cell culture types. MCP transcripts appear late in the FV3 lifecycle (Chinchar *et al.* 2011). Thus, entry into cells

and initiation of the viral life cycle appears not to be barrier to FV3 infecting rainbow trout cells.

After F3 addition, primary macrophage and RTS11 but not lymphocyte cultures developed cytopathic effects and apoptosis. FV3 appeared to target rainbow trout macrophages among leucocytes and to kill them by apoptosis. After FV3 exposure, both primary macrophage and RTS11 cultures developed cellular debris and experienced a decline in metabolism as measured with alamarBlue, an oxidation-reduction dye that is reduced by living cells, including lymphocytes (Petrenko *et al.* 2005). In contrast to macrophages, leucocytes in suspension remained viable, showing no change in metabolism upon exposure to FV3 for 24 h. Macrophage death appeared to be by apoptosis because within 24 h of FV3 introduction, both primary macrophage and RTS11 cultures showed DNA laddering and phosphatidyl serine (PS) externalization, which are hallmarks of apoptosis (Kroemer *et al.*
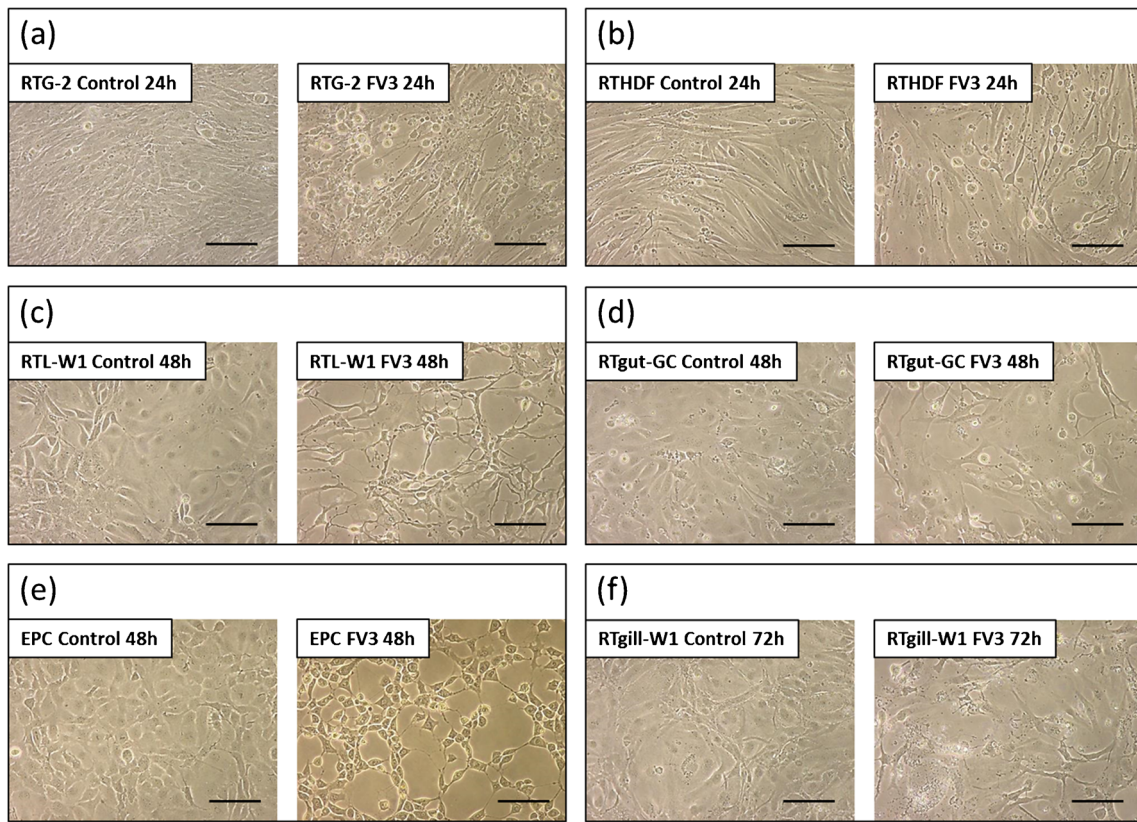
Figure 4. Observations of cytopathic effect (*CPE*) in adherent fish cell lines infected with FV3. All adherent cell lines showed CPE when infected with FV3 by up to 72 h as seen by damage in the continuity of the cell monolayer and/or cellular debris. However, the control cells showed no monolayer damage or debris. The *scale bar* represents 100 μm.
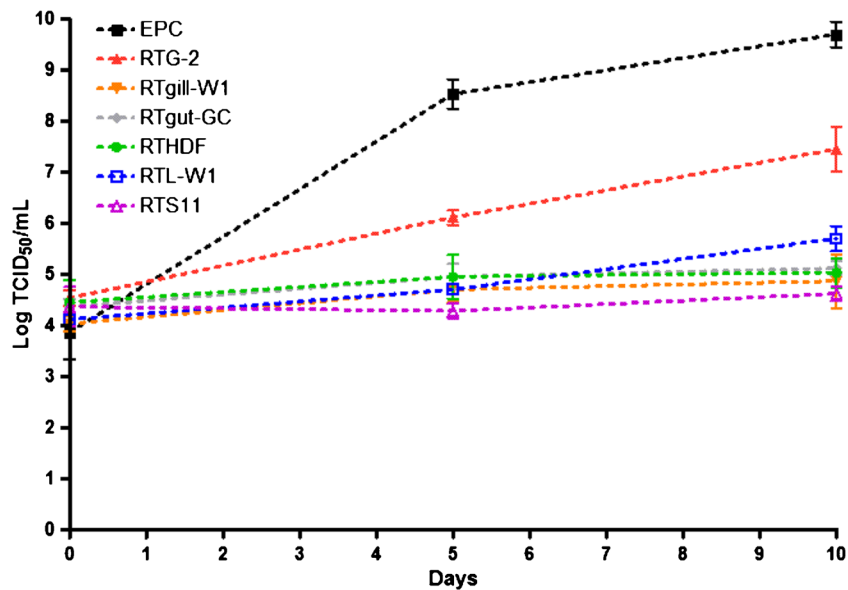

Figure 5. Titre of FV3 produced in rainbow trout cell lines and EPC. These cells were infected with FV3 and samples were taken from the supernatant of cell cultures at various time points for determining titre. EPC (*black line, shaded squares*), RTG-2 (*red line, shaded triangle*), and RTL-W1 (*blue line, empty square*) were the only three cell lines that produced an increase in FV3 titre by more than one log by day 10 pi when compared to day zero; EPC produced the highest titre, RTG-2 second highest while RTL-W1 least out of those three. For the rest of the cell lines, there was less than a 1 log change in titre between day zero and day 10 pi. The $TCID_{50}$/mL value for each time point was log transformed. *Data points* represent $N=3\pm SD$.

2009). Other studies hint at FV3/macrophage involvement. The injection of FV3 into rats specifically damaged liver macrophages (Gut *et al.* 1981). In the frog, *Xenopus laevis*, FV3 caused apoptosis in a small fraction of the macrophages but persisted in others, perhaps allowing *Xenopus* to be FV3 reservoirs (Robert *et al.* 2007; Morales *et al.* 2010; Grayfer *et al.* 2012; 2014). As FV3 killed most rainbow trout macrophages in vitro, these cells are unlikely to be in vivo locations of viral persistence but are possible sites of viral pathology in rainbow trout.

After FV3 addition, cultures of epithelial and fibroblast cell lines from rainbow trout developed CPE but not apoptosis. As well as killing rainbow trout macrophages by apoptosis, FV3 killed fibroblast and epithelial cells, but the cell death of the three epithelial and two fibroblast cell lines was not accompanied by DNA laddering and PS externalization and thus was non-apoptotic. Only a few reports have appeared on how ranaviruses kill fish epithelial and fibroblast cells. FV3 caused apoptosis in FHM cultures (Chinchar *et al.* 2003) and in a very small portion of the cells in EPC cultures (Holopainen *et al.* 2012). Possibly, apoptosis was not seen in the current study with EPC because a higher multiplicity of infection (moi) might have produced an amount of non-apoptotic death that overwhelmed the detection of a low level of apoptosis. Another Ranavirus, grouper iridovirus (GIV), induced apoptosis in EPC and the Chinook salmon embryo cell line, CHSE-214, but not in a grouper kidney cell line, GK (Pham *et al.* 2012). Therefore, ranaviruses appear to have the potential to kill fish cells in multiple ways. The mode of cell death that FV3 causes in fish fibroblast and epithelial cells will be interesting to investigate in the future.

FV3 production was nonexistent in most rainbow trout cell lines. Although high FV3 titres were consistently achieved in cultures of the fathead minnow cell line, EPC, titre increases were small or nonexistent in cultures of six rainbow trout cell lines, even though all cultures were held at a temperature optimal for rainbow trout cells (Bols *et al.* 1992). The FV3 titre produced on either RTG-2 or RTL-W1 was several orders of magnitude lower than on EPC. Not even a one-log increase in FV3 titre was seen with RTgill-W1, RTgut-GC, RTHDF, and RTS11. The possibility that a cell type(s) in vivo supports strong FV3 replication but is not represented in a rainbow trout cell line is difficult to rule out completely. Notwithstanding this, the results overall suggest that rainbow trout cells are unable to robustly produce FV3.

Why FV3 fails to replicate in rainbow trout cells, despite entering them, is open to two lines of speculation. Firstly, rainbow trout cells might lack something to adequately support the FV3 life cycle. One inadequacy might be in codon usage. Inefficient codon usage was experimentally observed for FV3 infection of mammalian cells (Eaton *et al.* 2008). If the codons carried by the FV3 genome were not the preferred or optimal codon used by rainbow trout then the inefficiency in translation may be the cause of low FV3 titre in rainbow cell cultures. Secondly, the antiviral mechanism of rainbow trout cells against FV3 might be more effective than those in cells of other species or better maintained in vitro. With this explanation, the ability of RTG-2, unlike the other rainbow trout cell lines, to produce FV3 might be accounted for by the antiviral mechanism in RTG-2 becoming impaired over the years that this line has been in culture. In support of this thought, prolonged cultivation of the baby hamster kidney cell line, BHK, led to strains that supported either rapid, slow, or no FV3 replication (Vilagines and McAuslan 1970).

One antiviral mechanism that might be crucial to determining whether cells produce virus is the timing of cell death. Ranaviruses code for anti-apoptotic factors, presumably to keep cells alive to support the early events of the viral life cycle, and apoptotic factors, to allow viral exit (Eaton *et al.* 2007). However, cells can respond to viruses by triggering cell death pathways early to prevent the completion of viral replication. Perhaps rainbow trout cells die, either by apoptotic or non-apoptotic modes, before significant FV3 amounts are produced. The particular mode of cell death might depend on the timing and kinds of interaction that occur between cellular apoptotic factors and viral anti-apoptotic factors. In this study, FV3 was produced in RTG-2 and not in RTHDF but both died by non-apoptotic modes. Pham et al. (2012) found that GIV was produced in GK and EPC but only EPC died by apoptosis. Thus, a correlation between how cells die and their ability to produce ranaviruses is not yet evident.

Conclusions

In all rainbow trout cell culture systems, including epithelial, fibroblast, and macrophage cell lines, FV3 caused CPE, except for primary lymphocyte cultures, which remained viable. Dying epithelial and fibroblast cell cultures did not express hallmarks of apoptosis and produced the virus poorly. Therefore, if FV3 were to breach the mucosal barriers of rainbow trout, the virus would be unlikely to replicate in the underlying epithelium and connective tissue. FV3 induced DNA laddering and PS externalization in macrophage primary cultures and in the macrophage cell line, RTS11. Thus, FV3 appeared to kill rainbow trout cells by at least two different mechanisms, possibly necroptosis in epithelial cells and fibroblasts and apoptosis in macrophages. If the FV3 were to get into the blood stream of rainbow trout, macrophages would likely die quickly, impeding dissemination and persistence of the virus, while other types of leucocytes might help clear the virus. Therefore, based on the response of cells in vitro to FV3, rainbow trout are not likely to be a reservoir host of FV3, and the risk of FV3 causing disease in wild rainbow

trout stocks appears to be low. Yet, the killing of rainbow trout cells by FV3 makes the consumption of FV3-infected amphibians by rainbow trout interesting to consider. Whether FV3 would be inactivated or blocked by innate antiviral mechanisms of the intestine or able to transitorily damage the intestinal epithelial barrier are interesting questions for future research.

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