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# CHARACTERIZING dsRNA PRODUCTION IN VIRUS-INFECTED FISH CELLS

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# CHARACTERIZING dsRNA PRODUCTION IN VIRUS-INFECTED FISH CELLS

by

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**ABSTRACT**

Viral dsRNA is produced by almost all viruses sometime during their replicative cycle. These viral nucleic acids are potent inducers of both innate and adaptive immune responses, and are therefore considered important immuno-modulators. Previous studies have shown that viruses produce dsRNA when replicating in mammalian cells; however, to date no one has demonstrated viral dsRNA production in virus infected fish cells. Therefore, the goal of this study is to investigate dsRNA production by fish viruses in fish cells, verifying production and performing initial characterization of the dsRNA molecules being produced. Three different rainbow trout cell lines were used in this study: rainbow trout gill (RTgill-W1, epithelial), rainbow trout gut (RTgutGC, epithelial) and rainbow trout gonad (RTG-2, fibroblast). These cell lines were selected because innate immune responses are relatively well characterized in RTG-2; while RTgill and RTgut represent two tissues that would be first to 'see' a virus infection *in vivo*. The study also includes three different fish viruses: viral haemorrhagic septicaemia virus (VHSV), which has a negative sense single stranded RNA (-ssRNA) genome, chum salmon reovirus (CSV), which has a double stranded RNA (dsRNA) genome, and frog virus3 (FV3), which has a dsDNA genome. These viruses were selected because they have different genomes and thus different replication cycles, which is important for verifying dsRNA production is not specific to one virus genome type. dsRNA production was measured using immunofluorescence, a technique which relies on J2, a mouse anti-dsRNA antibody. Not only does immunofluorescence with J2 verify that fish viruses produce dsRNA in fish cells, but it also indicates the location of dsRNA production

within the cell. An acridine orange stain was also performed to indicate the relative amount of dsRNA produced during a virus infection as well as the length of the dsRNA molecules to provide further evidence for dsRNA production by fish viruses in fish cells using an antibody-independent method. Because dsRNA is an important immuno-modulator, it has possible applications as a novel adjuvant for vaccines or as an antiviral therapy. The results from this study are important not only because it contributes to a better understanding of virus-host interactions, but characterizing viral dsRNA in fish cells could provide basic research evidence on which to build novel dsRNA-based therapies in fish.

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| | |
|---|---|
| **AqRV** | Aquareoviruses |
| **BHK** | Baby hamster kidney cells |
| **Bp** | Base pairs |
| **BSA** | Bovine Serum Albumin |
| **cDNA** | Complementary DNA |
| **CHSE-214** | Chinook salmon embryo cell line |
| **CPE** | Cytopathic effect |
| **CSV** | Chum salmon reovirus |
| **DNA** | Deoxyribonucleic acid |
| **dsDNA** | Double stranded DNA |
| **dsRNA** | Double stranded RNA |
| **EPC** | Epithelioma carp papulosum cyprinid cell line |
| **FBS** | Fetal bovine serum |
| **FHM** | Fathead minnow cells |
| **FITC** | Fluorescein isothiocyanate |
| **FV3** | Frog viruses |
| **IF** | Immunofluorescence |
| **INF** | Interferon |
| **IPNV** | Infectious pancreatic necrosis virus |
| **ISGs** | Interferon stimulated gene |
| **J2** | Monoclonal anti-dsRNA antibody |
| **Kb** | kilobase |
| **L-15** | Leibovitz's media |

| | |
|---|---|
| **MDA5** | Melanoma differentiation-associated gene 5 |
| **OAS** | OligoAdenylate Synthetase |
| **PAMPs** | Pathogen-associated molecular patterns |
| **PKR** | DsRNA-dependent protein kinase R |
| **PRRs** | Pattern recognition receptors |
| **poly IC** | Poly inosinic:poly cytidylic acid |
| **RNA** | Ribonucleic acid |
| **rRNA** | Ribosomal RNA |
| **RNAi** | RNA interference |
| **RIG-I** | Retinoic acid-inducible gene 1 |
| **RT- PCR** | Reverse-transcription polymerase chain reaction |
| **RTG-2** | Rainbow Trout gonadal fibroblast-like cell line |
| **RTgill-W1** | Rainbow Trout gill epithelia cell line |
| **RTgutGC** | Rainbow Trout gut epithelia cell line |
| **SR-AS** | Class A scavenger receptors |
| **-ssRNA** | Negative sense single-stranded RNA |
| **+ssRNA** | Positive sense single-stranded RNA |
| **TCID50** | Tissue culture infectious dose 50 |
| **TF** | Transcription factors |
| **TLR3** | Toll-like receptor 3 |
| **TRITC** | Tetramethylrhodamine isothiocyanate |
| **VHSV** | Viral hemorrhagic septicemia virus |
| **Vig** | Virus-induced genes |

# 1. GENERAL INTRODUCTION

## 1.1. Aquaculture and the Effect of Fish Viruses

Aquaculture, including water farming of both fish and shellfish, has developed and grown quickly as a provider of human food sources in many countries worldwide over the past 25 years; meanwhile, products from natural water have steadily declined (Meyer, 1991; U.S. Office of Aquaculture, 1986). In Canada, fish are very important for the Canadian economy; in 2007, aquaculture in Canada generated more than $1.0 billion (Fisheries & Oceans Canada, 2010). The greatest loss of farmed fish results from disease- an imperative factor causing economic loss. In 1988, the trout industry indicated that 50% of the 20.7 million trout produced were lost because of disease, meaning that approximately 1.04 x $10^6$ kg of trout fish were killed (USDA, 1989). Therefore, understanding the relationship between aquatic organisms and invading pathogens is very important for the development of methods to limit destructive agent such as viruses.

Rainbow trout and steelhead are caught wild or grown in aquaculture facilities. Viruses are a major threat to fish populations, including rainbow trout, and can spread extensively throughout fish farms. In fact, fish viruses devastate cultured-fish stocks, causing up to 100% mortality and resulting in significant financial loss (Kankainen *et al.*, 2005). For example, viral haemorrhagic septicaemia virus (VHSV) is a particularly devastating fish virus in Canada (Martinez-Lopez *et al.*, 2012).

## 1.2. Fish Viruses Used in this Study

VHSV is a member of rhabdoviridae family. It has a negative sense single-stranded RNA (-ssRNA) genome composed of an 11-kb unsegmented genome. The genome has six genes: the nucleocapsid protein (N), polymerase-associated 6++phosphoprotein (P), matrix protein (M), surface glycoprotein (G), a unique non-virion protein (NV), and virus polymerase (L) (3′-N-P-M-G-NV-L-5’) (Schütze *et al*, 1999; Chang *et al.,* 2011). A fish infected with VHSV appears to be listless, limp, and exhibits irregular swimming behaviour (Kenyon & Dept, 2012). Negative effects of VHSV on marine or freshwater fish have been documented for more than 50 species; in the Great Lakes alone genotype IV-b has been shown to infect at least 28 fish species (Winton *et al.,* 2008; Crane & Hyatt, 2011). VHSV, particularly strain IV-b which was used in the present study, induces mild cytopathic effects (CPE) in rainbow trout cell lines (Pham *et al.,* 2013).

Chum salmon reovirus (CSV) was the first member of the aquareoviridae (AqRV) family to be isolated from salmonids (Winton *et al*., 1981). CSV has a double-stranded RNA (dsRNA), segmented genome, containing 12 proteins. The segments range from 18.2 to 30.5 kb in size (Winton *et al.,* 1981). Cytopathic effects (CPE) have been observed in CSV-infected salmonid epithelial, fibroblast, and macrophage cell lines, where it induces the formation of syncytia, whereby cells fuse together to form large cells with many nuclei (DeWitte-Orr & Bols, 2007).

Frog virus (FV3) is a species of the ranavirus genus of the family Iridoviridae. FV3 has a large double-stranded DNA genome ranging from 105 to 140 kb (Rothenburg *et al.,* 2011; Chinchar *et al.,* 2011 ), and its genome is replicated in both the nucleus, where the first stage of DNA synthesis takes place, and the cytoplasm, where the second stage of DNA synthesis and viral assembly site occur. FV3 can infect a variety of vertebrates such as amphibians, reptiles, and teleost fish (Eaton *et al.,* 2008), as well as being able to replicate and produce a productive infection in multiple cell lines derived from simian, rodent, piscine, and avian animals (Granoff, 1969).

Both VHSV and CSV are able to induce innate antiviral immune responses in fish cells (Table 1) (DeWitte-Orr *et al.,* 2007, Tafalla *et al*., 2007, Tafalla *et al*., 2008, Chang *et al.,* 2011). Some of the antiviral genes which are activated during VHSV and CSV infections include those induced by dsRNA in mammals, namely: Retinoic acid-inducible gene I (RIG-I), Melanoma differentiation-associated gene5 (MDA5), Mx proteins, and virus-induced genes (vig). Less is known regarding FV3-induced innate antiviral responses. However, it has been shown that FV3 encodes a translation initiation factor that acts as an inhibitor for dsRNA-induced host antiviral mechanisms (Essbauer *et al.,* 2001). Moreover, family members of this virus are able to induce antiviral genes in fish (Wu *et al.,* 2012). This suggests that not only do fish cells possess the ability to respond to viral dsRNA, but that fish viruses used in this study are likely capable of making dsRNA.

## 1.3. How Viruses Produce dsRNA

Viruses with ssRNA genomes produce dsRNA via a replicative intermediate, as both sense and antisense directions are transcribed from their genomes (Figure 1). For viruses with dsRNA genomes, the genomes remain in the capsid during the entire viral life cycle, positive sense ssRNA genome copies leave the capsid, are used for viral protein translation and are packaged into subviral proteins where their negative sense complement is synthesized to make dsRNA (Jacobs & Langland, 1996) (Figure 1). At no time should dsRNA be out of a capsid; however, during an actual virus infection unpackaged or naked genomes may be released in the infected cell, thereby activating dsRNA-dependent enzymes. For DNA viruses, many contain genomes with overlapping genes, or genes in both directions; therefore, complementary mRNAs are produced from transcribed genes in opposing directions, or from overlapping transcription of mRNAs genes, and these transcripts self-anneal to produce dsRNA (Jacobs & Langland, 1996) (Figure 1).

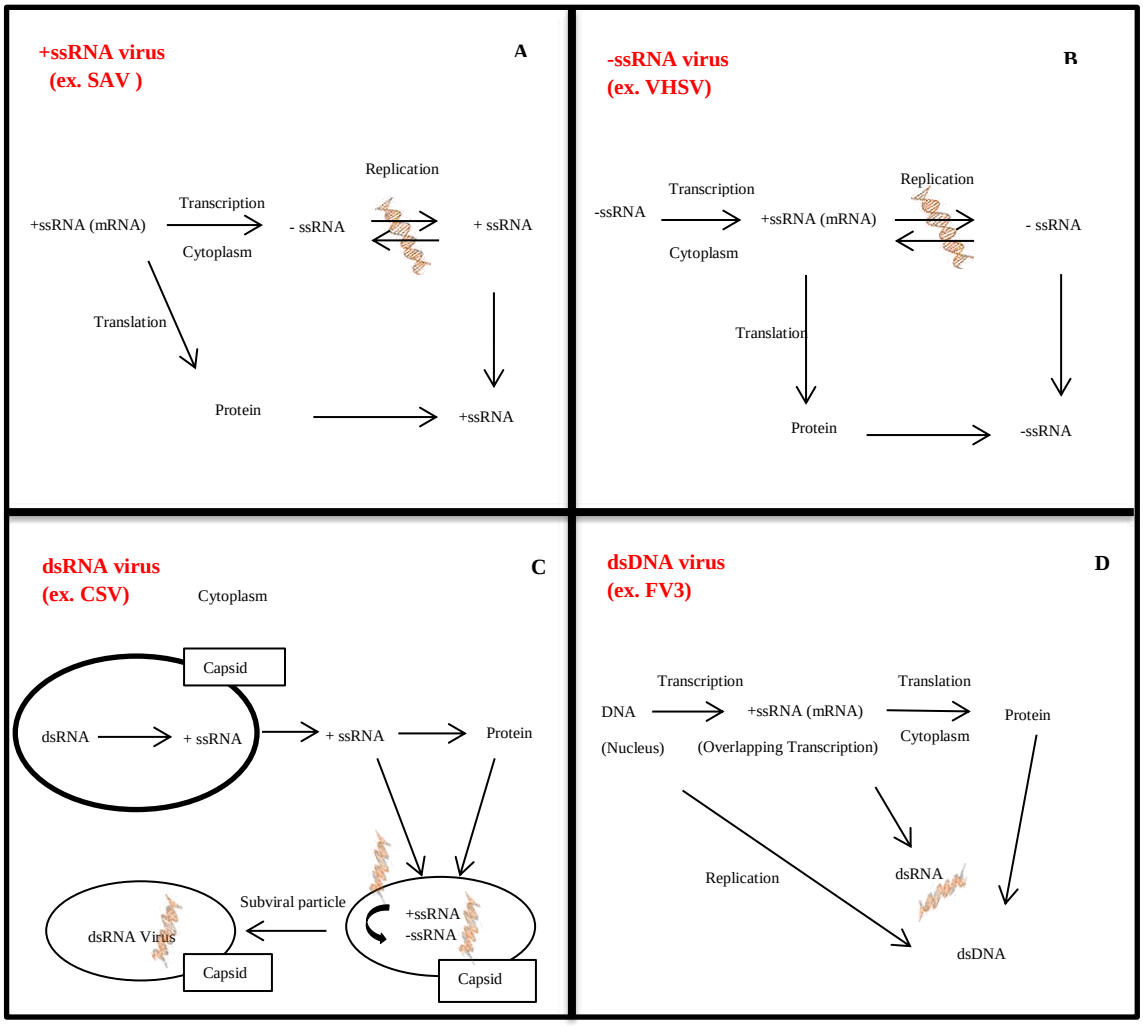


**Figure 1: Virus genome replication and production of dsRNA.** Viruses with (+) RNA genomes copy their genomes via a (-) RNA intermediate, while (-) RNA genomes replicate via (+) RNA intermediate (A&B). Both RNA viruses (negative and positive strands) produce dsRNA once the complementary strand is produced, and then these molecules self-anneal to make dsRNA. The genomes of dsRNA virus (C) remain safely within the viral capsid throughout its life cycle. It is only if the dsRNA is mis-packaged and cytoplasmic that cell can sense the dsRNA. Viruses with dsDNA genomes are transcribed to mRNA. Occasionally transcription makes many complementary mRNAs, which anneal to make dsRNA (D). Single-stranded genomes are labelled as plus and minus, with the plus strand being the same sense as mRNA and the minus strand having a sequence complementary to mRNA.

## 1.4. Viral infection and Innate Immune Mechanisms

### 1.4.1. Viral dsRNA

Viral dsRNA acts as a signal indicating the presence of a viral infection and is a potent inducer of antiviral responses. dsRNA is sensed by the infected cell during virus replication and by neighbouring cells following cell lysis of the infected cell and release of viral dsRNA into the extracellular space. Class A scavenger receptors (SR-As) are host cell surface receptors involved in extracellular dsRNA entry (Figure 2). These receptors bind extracellular dsRNA and deliver it to intracellular sensors such as toll-like receptor 3 (TLR3) in the endosomes, and retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) sensors in the cytoplasm (DeWitte-Orr *et al.*, 2010). When these intracellular sensors bind dsRNA, they activate pathways that culminate in the expression of type I interferons (IFN) and IFN-stimulated genes (ISGs), the actions of which block virus replication in infected cells (Robertsen, 2008) (Figure 2). Interferons can also activate immune responses in neighbouring uninfected cells and stimulate the accumulation of ISGs in the uninfected cell; this is called an antiviral state. Viral dsRNA is not only sensed in the cell in which it was generated, but also by neighbouring cells as mentioned above (DeWitte-Orr & Mossman, 2010). To understand the viral dsRNA molecule, it is important to investigate and elucidate dsRNA structure, biological functions, and physical properties. Viral dsRNA molecules are long (> 40 bp), composed of an antiparallel helix that has a narrow major and minor deep groove. Its minor groove has the ability to bind proteins since it possesses 2´-hydroxyl groups

(Dewitt-Orr & Mossman, 2010). dsRNA is more stable than ssRNA; it is relatively resistant to nuclease activity due to its unique structure. dsRNA is not degraded by RNase A or B because it cannot be bound by ssRNA nucleases; however, is can be degraded with RNase III (DeWitte-Orr & Mossman, 2010).

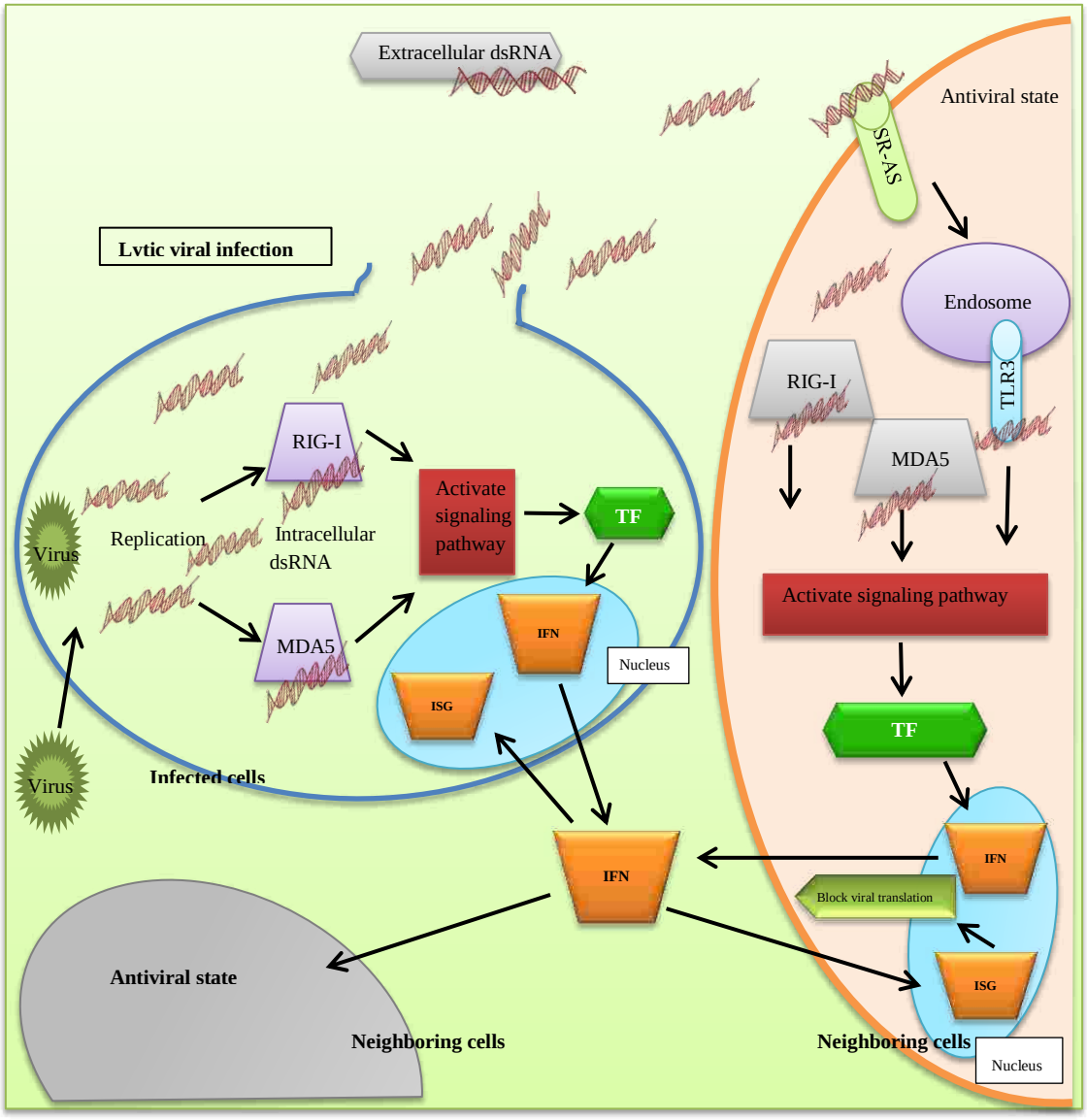
Extracellular dsRNA
Antiviral state
SR-AS
Lvtic viral infection
Endosome
TLR3
RIG-I
MDA5
Activate signaling pathway
TF
Virus
Replication
Intracellular dsRNA
Activate signaling pathway
TF
IFN
Nucleus
ISG
Virus
Infected cells
IFN
IFN
Block viral translation
ISG
Antiviral state
Neighboring cells
Neighboring cells
Nucleus

**Figure 2: Summary of the dsRNA production pathway and innate immune responses of the cell.** dsRNA is generated in the intracellular space of a virus-infected cell. Intracellular dsRNA is sensed by the cytoplasmic sensors (RIG-I and MDA5). These sensors activate signalling pathways that culminate in the activation of transcription factors (TF) which stimulate interferon (IFN) production. IFN is released from the cell. It acts in an autocrine or paracrine fashion, signaling through the IFN receptor to induce interferon-stimulated gene (ISGs) in the infected cell to block the viral infection, and also induces ISG production and the establishment of an antiviral state in neighbouring (uninfected) cells. dsRNA can be released into the extracellular space, from virus-infected cells during cell lysis, and be sensed by surface-expressed class A scavenger receptors (SR-As) delivering the dsRNA to endosomal TLR3, which will also trigger the production of IFNs, ISGs and the antiviral state in neighbouring cells (Modified from DeWitte-Orr & Mossman, 2010).

### 1.4.2. Stimulating Antiviral Genes and Antiviral Defence

The innate antiviral immune response is able to inhibit and control a viral infection. It is considered the first line of defence against viral pathogens, and results in the activation of adaptive immune responses. This immune response depends on the detection of viral pathogen-associated molecular patterns (PAMPs) by host expressed pattern recognition receptors (PRRs), which in turn up-regulate the expression of interferon (IFN) and interferon-stimulated genes (ISGs) (DeWitte-Orr & Mossman, 2010). In this current study we discuss what is known regarding PRRs, IFN and ISGs.

#### *1.4.2.1. Pattern Recognition Receptors (PRRs)*

Viral dsRNA is arguably the most important pathogen-associated molecular pattern (PAMP) associated with viral infections, since cells express a number of proteins to detect dsRNA. Intracellular viral dsRNA is sensed by the endosomal PRR Toll-like Receptor 3 (TLR3) and cytoplasmic sensors RIG-I-like receptors (RIG-I and MDA5), dsRNA dependent protein kinase (PKR), and oligoadenylate synthetase (OAS). TLR3 is an endosomal sensor that functions by binding dsRNA lengths of 39-48 bp (Leonard *et al*., 2008). Essentially, the more length that is bound by TLR3, the more immune responses are created. TLR22 is a member of PRRs that has been shown only in aquatic species, multiple fish species, and amphibians (Matsuo *et al.*, 2008). TLR22 is located on the cell surface and functions as an extracellular sensor for longer dsRNA extracellular (~1 kb) to protect aquatic organisms from viruses in the external environment.

The retinoic acid-inducible gene I (RIG-I) - like receptors (RLRs) - is a family of dsRNA PRRs including RIG-I and melanoma differentiation-associated protein 5 (MDA5). RIG-I and MDA5 are cytoplasmic helicases located in the cytoplasm of host cells (Takeuchi & Akira, 2008; Peisley & Hur, 2012). Both of these PRRs recognize viral dsRNAs; however, they distinguish between lengths of dsRNAs (length-dependent recognition). RIG-I identifies shorter segments (<1 kb) and MDA5 identifies longer segments (>2 kb) of viral dsRNAs (Kato *et al.,* 2008).

The dsRNA-dependent protein kinase (PKR) is a member of a small family of kinases that function to control cellular translation by phosphorylating the translation initiation factor eIF2α (DeWitte-Orr & Mossman, 2010). PKR contains double-stranded (ds) RNA binding domains (dsRBD) and a kinase domain (Rothenburg *et al.,* 2011). The mechanism of PKR activation is thought to occur through an interaction between dsRBD of PKR and the dsRNA helix. Longer dsRNA strands (>30 bp) are required to activate PKR and inhibit viral transcription by phosphorylating the translation initiation factor eIF2α. PKR reduces the translation of all mRNAs in the cell, thereby preventing viral protein synthesis.

The oligoadenylate synthetase (OAS) is another cytoplasmic PRR sensor that binds long dsRNA and requires a minimal length of 18-20bp dsRNA for activation. OAS is an important IFN-induced protein that has yet to be cloned in fish (Robertsen, 2006). The mechanism of OAS in binding viral dsRNA is well understood in mammals. When OAS binds dsRNA, ATP is converted into 2′, 5′ -linked oligoadenylates; cytoplasmic RNase L is activated by binding 2′, 5′- linked oligoadenylates, degrading viral and

cellular ssRNA molecules and causing blockage of both host and virus protein synthesis (DeWitte-Orr & Mossman, 2010; Robertsen, 2006).

#### *1.4.2.2. Interferons*

Interferons (IFNs) are cytokines. There are two categories of IFNs - type I and type II. Typically, type I IFNs are induced in innate antiviral mechanisms, whereas type II IFNs stimulate T cells, adaptive immunity, and antibacterial immunity (Decker *et al.*, 2005). Rainbow trout IFNs are divided into two classes. Class 1 contains IFNs with two cysteine residues and a single disulphide bond: rtIFN1, rtIFN2, and rtIFN5 (Chang *et al.*, 2009). Class 2 consists of IFNs having four cysteine residues and two disulphide bonds: rtIFN3 and rtIFN4 (Purcell *et al*., 2009; Chang *et al.*, 2009). dsRNA (native dsRNA) in general and poly IC (a synthetic dsRNA) in particular are able to induce IFN and activate an antiviral response in fish cells (Eaton, 1990; DeWitte-Orr & Mossman, 2010). The significant function of interferon production is to inhibit all stages of viral replication including transcription, RNA stability, initiation of translation, assembly, and release (Stark *et al.*, 1998) by stimulating the expression of ISGs and establishing an "antiviral state" not only in the infected cells but also in neighboring healthy cells (uninfected).

#### *1.4.2.3. Interferon Stimulated Genes*

Interferon stimulated genes (ISGs) are interferon inducible factors that inhibit virus replication and regulate cell cycle and cell death (Goodbourn *et al.*, 2001). ISGs are

found in an inactive form within the cell until the dsRNA is detected. A few ISG have been identified in fish, including Mx1-3 and vig1-10 (O'Farrell *et al*., 2002). Mx proteins are dynamin-related members of the large GTPase super-family (Lee *et al.*, 2002). It has been shown that these proteins are produced in many vertebrate species including mice (Lindenmann, 1962) and teleost fish (Lee *el al*., 2002) by type I interferon, dsRNA, or viral infection. When these genes are expressed, they can be at high levels which can be used to indicate type I interferon expression (Horisberger, 1995). In addition to fish, Mx has been identified in a number of mammals, chicken, and invertebrate species such as mollusks and abalone (Schumacher *et al*., 1994; De Zoysa *et al*., 2007). Mx, Mx1, and Mx3 have been cloned in rainbow trout. Mx1 and Mx3 are cytoplasmic genes, whereas Mx2 is located in the nucleus (Leong *et al.*, 1998). Previous studies have shown a direct antiviral role for Mx; for instance, Atlantic salmon Mx1 protected the Chinook salmon embryonic cell line (CHSE-214) against ISAV and IPNV (Larsen *et al*., 2004; Kibenge *et al*., 2005a). Another group of ISGs, the virus-induced genes (vigs), were initially identified from the head kidney of VHSV-infected rainbow trout (Boudinot *et al.*, 1999).

## 1.5. Rainbow Trout Cell Lines

Rainbow trout (*Oncorhynchus mykiss*) is a member of the salmon family. As a cold water teleost fish, it is native to the rivers and lakes of North America, and is one of the most popular freshwater fish farmed in Canada (Fisheries and Oceans Canada). Fish cell lines were established in the 1960s with the development of rainbow trout gonad cell lines (RTG-2) by Wolf and Quimby (1962). The history of rainbow trout cell lines for

studying antiviral immune responses in specific cell types is valuable and extensive; as rainbow trout are susceptible to many aquatic viruses. Rainbow trout cells have been infected by fish viruses such as IPNV, VHSV, and CSV; moreover, they have caused CPE and have activated IFN and ISGs in rainbow trout cell lines (DeWitte-Orr & Bols, 2007; Tafalla *el al*., 2007; Chang *et al*., 2011; Pham *et al*., 2013). Fish in general, and rainbow trout in particular, possess dsRNA sensors and dsRNA-induced immune genes; thus, rainbow trout have been used in this study to determine whether dsRNA is made by fish viruses. Many cell lines have been derived from rainbow trout tissue such as RTG-2, rainbow trout liver (RTL-W1), and rainbow trout spleen (RTS11). In this study, three rainbow trout cell lines were used: two rainbow trout epithelial cell lines (RTgutGC and RTgill-W1) and rainbow trout fibroblastic cell line (RTG-2). RTgutGC was developed from the intestine and RTgill-W1 was developed from the gill of *Oncorhynchus mykiss* (Kawana *et al*., 2010; Bols *et al*., 1994). RTG-2 is derived from gonad tissue, and was chosen as it is one of the best characterized rainbow trout cell lines available; furthermore, its antiviral immune responses are somewhat known. RTgutGC and RTgill-W1 were chosen because they represent the two tissues that act as barriers between the fish and its environment and would be the first cells within the fish to be infected.

# 2. RESEARCH OBJECTIVES AND HYPOTHESIS

Fish viruses, similar to mammalian viruses, can have either single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), or dsDNA genomes (Table 1& 2). To our knowledge, no one has shown that fish viruses make dsRNA during a replicating infection in fish cells. We hypothesized that fish viruses must make dsRNA as fish cells have been shown to have many of the same dsRNA sensors and dsRNA-induced genes (Table 1) as mammals (Table 2). Thus, this study aims to investigate the ability of three fish viruses with different genome types to produce dsRNA in three rainbow trout cell lines.

The present study contains two objectives:

1) Demonstrate whether fish viruses produce dsRNA in fish cells; and

2) Characterize the viral dsRNA molecule being produced, including its length and localization within the cell

Table 1: Evidence for dsRNA-induced mechanisms in fish. Although no one has shown directly that dsRNA is produced during a viral infection in fish cells, there is evidence in the literature that viruses with different genomes, infecting different fish species, are able to mount a dsRNA-mediated antiviral response.

| **Genome** | **Fish viruses** | **Whole fish or fish cells** | **Antiviral genes** | **References** |
|---|---|---|---|---|
| (+) ssRNA | Salmon alphavirus (SAV) | Atlantic salmon (TO cells) | Retinoic acid-inducible gene 1 (RIG-I), Melanoma differentiation-associated gene 5 (MDA5), Laboratory of Genetics and Physiology 2 (LGP2) | Chang *et al.*,2011 |
| (-) ssRNA | Infectious hematopoietic necrosisvirus (IHNV) | Rainbow trout gonad (RTG-2) & Chinook salmon embryo (CHSE-214) | Mx proteins | Trobridge *et al.*,1997 |
| | Viral haemorrhagic septicaemia virus (VHSV) | Rainbow trout gonad (RTG-2) & Epithelioma papulosum cyprinid (EPC) | Mx1, Mx2& Mx3 | Tafalla *el al.*, 2007 |
| | | Rainbow trout spleen (RTS11) | Mx1, Mx2& Mx3 | Tafalla *el al.*, 2008 |
| | | Rainbow trout gonad (RTG-2) & Rainbow trout spleen (RTS 11) | Retinoic acid-inducible gene 1 (RIG-I), Melanoma differentiation-associated gene 5 (MDA5), RIG-I-like Receptor (LGP2) | Chang *et al.*, 2011 |
| dsRNA | Infectious pancreatic necrosis virus (IPNV) | Atlantic salmon (TO cells) | Mx protein & interferon stimulated genes (ISG15) | Robertsen, 2008 |
| | | Rainbow trout gonad (RTG-2) | Toll-like receptor (TLR22) | Matsuo *et al.*, 2008 |
| | | Rainbow trout gonad (RTG-2), Zebrafish & Chinook salmon embryo (CHSE-214) | dsRNA-dependent protein kinase (PKR) | Garner *el al.*, 2003 |
| | Chum salmon reovirus (CSV) | Rainbow trout gonad (RTG-2) & Rainbow trout spleen (RTS 11) | Mx proteins, Virus induced gene-1 (Vig-1) | DeWitte-Orr *et al.*, 2007 |
| DNA | Frog virus 3 | N/D | N/D | N/D |
| | *Iridovirus* family members | Orange-spotted grouper | IgM, Mx-1& TNF-a | Wu *et al.*, 2012 |
| | Ranavirus | Zebrafish | Inhibitor of the PKR | Essbauer *et al*, 2001 |

**Table 2: Evidence for dsRNA production by viruses in mammals.** In mammals it has been shown that viruses with different genome types are all able to produce dsRNA. The location of dsRNA production correlates with the virus replication cycle and can be detected using a number of different research methods (Adapted from DeWitte-Orr & Mossman, 2010).

| Genome | Virus | dsRNA replication | Detection method | Cells studied | References |
|---|---|---|---|---|---|
| (+) ssRNA | (WNV) west Nile virus : (Kunjin virus) | Cytoplasm | Immunofluoresence (IF), polyclonal antibody, immunoblot using monoclonal antibody (J2) | Mouse embryonic fibroblast (MEFs) +Vero cells | DeWitte-Orr *et al*., 2009 |
| | Rubella & SFV | | IF, TEM polyclonal antibody | Vero cells | Lee *et al.*, 1994 |
| | Sindbis virus | | IF, polyclonal antibody | N/D | Stollar & Stollar,1970 |
| | SARS-COV coronavirus | | IF, monoclonal antibody (J2) | Vero cells, Hela cells & Baby hamster kidney (BHK-1) cells | Weber *et al.*, 2006 |
| | Encephalomyocarditis virus (EMCV) | | Sedimentation rate and nuclease resistance, IF, immunoblot, monoclonal antibody (J2) | MEFs | Weber *et al.*, 2006 |
| | Hepatitis C virus (HCV) | | FISH, monoclonal antibody (J2), | Huh-7 cells | Targett-Adams *et al.*, 2008 |
| | HIV1 | Nucleus | Activation of dsRNA-dependent proteins | N/D | Silverman & Sengupta, 1990 |
| (-) ssRNA | Vesicular stomatitis virus (VSV) | Cytoplasm | IF, immunoblot, monoclonal antibody (J2) | MEFs | Kato *et al.*, 2008 |
| dsRNA | Reovirus | Cytoplasm | IF, monoclonal antibody (J2) | Vero cells | Weber *et al.*, 2006 |
| DNA | SV40 | Nucleus | Nuclease resistance | monkey cells | Aloni, 1972 |
| | Adenovirus (Adv) | | IF, monoclonal antibody (J2), Nuclease resistance | Hela cells & BHK cells | Weber *et al.*, 2006 |
| | Herpes simplex virus (HSV1) | | IF, monoclonal antibody (J2), Nuclease resistance, Tm | Vero cells, Hela cells, BHK cells & HEp-2 | Weber *et al.*, 2006; Jacquemont & Roizman, 1975 |
| | Vaccinia Virus (VAC) | Cytoplasm | IF, monoclonal antibody (J2), Nuclease resistance | Hela cells & BHK cells, Chick embryo cells | Weber *et al.*, 2006 |

# 3. MATERIALS AND METHODS

## 3.1. Cell culture

Three fish cell lines were used in this study. Rainbow trout gonad (RTG-2, fibroblast), rainbow trout gill (RTgill-W1, epithelial) and rainbow trout gut (RTgutGC, epithelial) were obtained from Dr. Niels Bols' lab at the University of Waterloo. All of the three rainbow trout cell lines were passaged and maintained in T75 $cm^2$ flasks (Falcon, Bedord, MA) with 10 ml cell culture medium containing 10% fetal bovine serum (FBS, Sigma, St. Louis, USA) and 1% penicillin/streptomycin (Fisher Scientific) , in L-15 (Fisher Scientific, Fair Lawn, New Jersey, USA), and incubated at room temperature. Cells were spilt at confluency (approximately 75 - 80%). For virus infection experiments (IF), cells were plated into 12 well tissue culture plates (Falcon, Corning, NY) with glass coverslips, and incubated at room temperature overnight. Following overnight incubation, the old medium was removed and cells were infected with the appropriate virus.

## 3.2. Virus propagation and infection

CSV was obtained from Dr. Niels Bols' lab and propagated on Chinook salmon embryo cell lines, CHSE-214 ($TCID_{50}$= 1.58X $10^4$/ml). CHSE-214 cells were incubated with 1 ml CSV prep and 9 ml of fresh media for seven days. CSV containing medium (CCM) was filtered using a 0.22 um filter (Thermo Scientific, New York, USA) and kept frozen at -80$^o$C until usage. CHSE-214 was plated into 96 well plates (3x $10^4$ cells/well)

(Falcon, Franklin Lakes, NJ), viral suspensions were diluted from $10^{-1}$ to $10^{-6}$, and 200 µl was added to each well (6 wells/dilution). After a seven-day incubation period at room temperature, $TCID_{50}$/ml values were calculated using the Reed and Muench method (Reed & Muench, 1988). $TCID_{50}$ is the virus dose that causes 50% death. The same method was followed with VHSV and FV3. VHSV-IVb (strain 0771) and FV3 were obtained from Dr. Bols' lab and were propagated on monolayers of an epithelial cell line, EPC. VHSV $TCID_{50}$ was 3.16X $10^5$/ml, while FV3 was 1.99 X $10^6$/ml. For VHSV, cells were infected with serial dilutions of the virus beginning with a viral titre of $10^5$ tissue culture infections dose $(TCID)_{50}$/ml, for FV3 and CSV the stock virus titre was used for subsequent dilutions. Viral suspensions were diluted from $10^{-1}$ to $10^{-5}$ and six wells were inoculated with 450 µl of each dilution. These cultures were then incubated for the appropriate time for each virus and cell type at room temperature. Following this period, the cells were fixed and prepared for immunofluorescence. For the acridine orange stain experiments, cells were infected with the determined optimal viral dilutions for five days, and total RNA was extracted and loaded onto a 1% agarose gel (0.4 g agarose, 40 ml 1x TAE buffer ); 1x TAE buffer was made from 20 ml 50x TAE buffer + Milli-Q $H_2O$ up to 1000 ml .

## 3.3. Antibodies

Mouse anti-dsRNA antibody (J2), the primary antibody that was used in this study, was obtained from English and Scientific Consulting, Hungary. 200 µg of J2 was reconstituted (200 µl sterile Milli-Q $H_2O$ + 20 µl of 10 mg/ml BSA) to have a final

concentration of 10 mg/ml. This antibody was stored at 20$^{o}$C. The secondary antibody was a goat anti-mouse (AlexaFluor488) (Sigma) used at 1:200 dilutions for immunofluorescence experiments. The antibodies were diluted with block solution (1x PBS 50 ml (Fisher Scientific), 2 % goat serum 1 ml (Sigma), 0.03 g/ml BSA 1.5 g (Fisher Scientific), and 0.02 % Tween-20 10 µl (Fisher Scientific). 40 µl was added to each well and incubated for one hour in the dark.

### 3.4. Immunofluorescence analysis

Cell cultures were prepared as described in Section 2.1. Virus stocks were serially diluted ($10^{-1}$–$10^{-5}$) in L-15 growing media, and added to each culture. Following seven, five, three, and two days incubation at room temperature (20$^{o}$C) (number of incubation days dependent on cell type and virus), cells with differential viral dilutions were fixed with 10% formalin (500 µl/ well) (Fisher Scientific) and permeabilized with 100 ml PBS, and 100 µl Triton x-100 (0.001 %) (Fisher Scientific) for 10-15 min at room temperature. Cells were then washed three times with PBS. Next, block solution was added and cells were incubated overnight at 4$^{o}$C. 1:200 dilution of primary antibody (J2, mouse anti-dsRNA) and 1:200 dilution secondary antibody (goat anti-mouse) were added and incubated for 45 minutes to one hour for each addition. Cells were then stained with DAPI, 4′, 6-diamidine-2-phenylindole, 30 µl 10 mg/ml DAPI (Biotium, Hayward, CA) and 3 ml PBS in order to fluorescently stain nuclei. Coverslips were mounted to slides using Polyscience's Glycerol, p-phenylenediamine, carbonate-bicarbonate buffer, and dsRNA was visualized using fluorescence microscopy (Figure 3). For time course

experiments, cells were plated into 12 well tissue culture plates with coverslips and incubated overnight. Next, the cells were infected with VHSV ($10^2$ $TCID_{50}$/ml), or FV3 ($10^3$ $TCID_{50}$/ml) and they were incubated for one to five days. Also, cells were infected with CSV ($10^1$ $TCID_{50}$/ml) for one to seven days. Immunofluorescence was performed as described above. The three fish viruses VHSV-IVb, CSV, and FV3 were tested with all three rainbow trout epithelial and fibroblast cell lines (Appendix A). The optimal dilutions for the time course experiments were chosen based on positive results in the virus titre experiments.

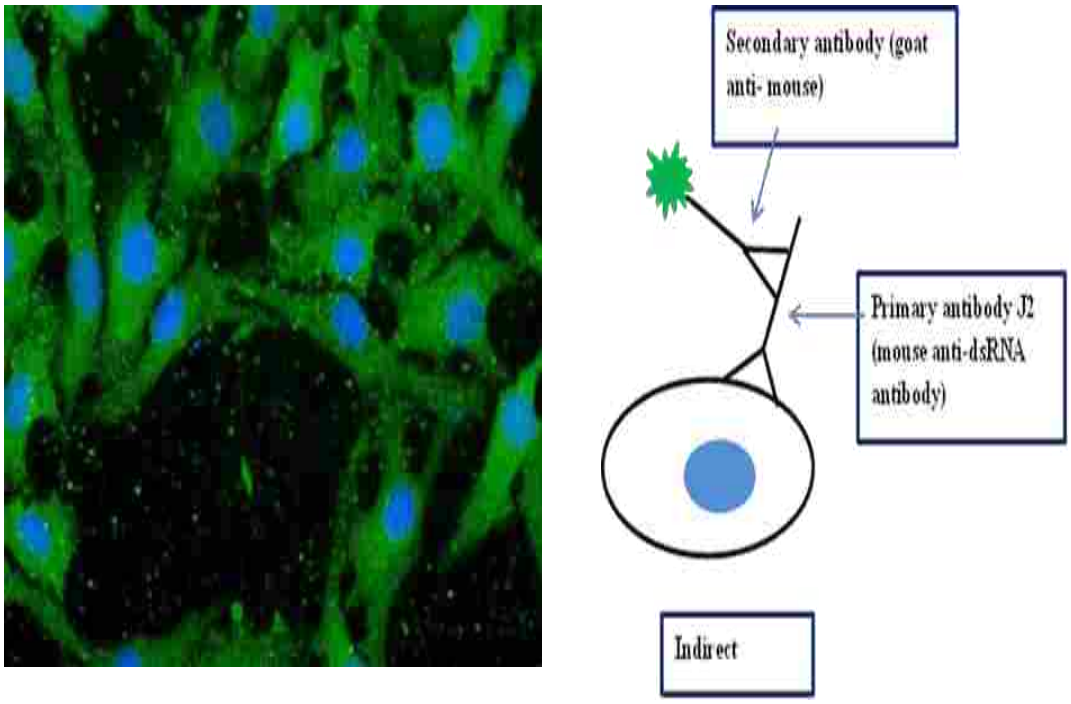


**Figure 3: Immunofluorescence method to detect dsRNA in rainbow trout cell lines.** RTgill-W1 is infected with VHSV for 5 days in a 12 well culture plate, then treated with the monclonal antibody J2 and the secondary goat anti-mouse antibody conjugated to AlexaFluor 488 (left); The blue stain (DAPI) indicated the cell nucleus while the green stain (FITC) indicates the location of dsRNA. The Figure shows the indirect IF method that has been used to bind viral dsRNA in fish cell lines (RTgill-W1), where the primary antibody (J2) binds to dsRNA, the secondary antibody binds the Fc domain of the primary antibody, and the secondary antibody is conjugated to a fluoresent dye which is visualized using fluorescence microscopy.

## 3.5 Immunofluorescence Microscopy and Nikon NIS-ELEMENTS Software

Cells were prepared for visualizing using a fluorescence microscope and a fluorophore-labeled secondary antibody. Immunofluorescence was performed in detecting and visualizing viral dsRNA with J2 antibody through indirect immunofluorescence (Figure 3). Fluorescein isothiocyanate (FITC) was used to detect dsRNA in the cell. DAPI (nuclei stain) and FITC (dsRNA stain) pictures were taken separately. Nikon NIS-ELEMENTS software was used to merge DAPI and FITC images. To quantify dsRNA production, dsRNA production was measured from IF intensity for each figures by selecting ROI area and making an automated measurement. The mean intensity data was chosen and exported to an Excel file. The IF intensity for each figure was then divided by the number of cells. Cells number was counted manually. Next, the statistics were plotted on graphs for virus titres experiments (Figures 6, 7 & 8) and for time points experiments (Figures 10, 11 & 12).

## 3.6. Acridine Orange Stain (AO)

### 3.6.1. RNA extraction

Cells were plated into T75 $cm^2$ flasks (approximately 85% confluency) and incubated overnight at room temperature. Cells were infected with the optimal viral titre determined for VHSV ($10^2$ $TCID_{50}$/ml) for five days. RNA from uninfected and infected cells was isolated using Trizol (Life technologies, USA), and then 80 µl chloroform (Fisher Scientific) was added and centrifuged at a maximum speed at $4^oC$. The clear

phase was removed in new tubes, 200 µl isopropanol alcohol (BDH, West Chester, PA) was added, incubated at room temperature for 10 min and was then centrifuged for 10 min at 4$^{o}$C. The supernatant was removed, 400 µl 75% ethanol (37.5 ml ETOH+ 12.5 ml Milli-Q $H_2O$) was added to the pellet, and centrifuged for five minutes at 4$^{o}$C. The liquid phase was entirely removed and the pellet was left to air dry for 10 min. Next, 10 µl DNA quality $H_2O$ was added to the pellet and incubated at 55$^{o}$C for 10 min. Total RNA was quantified using a NanoDrop spectrometer. Samples were stored at -80$^{o}$C.

### 3.6.2. AO gel stain

One microgram of total RNA was loaded onto a 1% agarose gel (Fisher Scientific) and the gel ran at 70 V for 75 min. A 1 kb DNA ladder was used as a size marker (Fermentas, CA). Gels were stained for 10 min with 30% acridine orange dye (Fisher Science) (7.5 mg acridine orange dye dissolved in 250 ml Milli-Q $H_2O$). Then, gels were destained under hot running water for 20 minutes followed by cold running water for five min. Finally, gels were destained into Milli-Q $H_2O$ overnight in the dark. Gels were imaged using UV transillumination.

# 4. RESULTS

## 4.1. Determining dsRNA production of fish viruses in fish cell lines

### 4.1.1. Viral dsRNA production in fish cells infected with increasing virus titres

The present study shows that all three fish viruses - VHSV, CSV, and FV3 – that were tested into rainbow trout cell lines: RTgill-W1, RTgutGC, and RTG-2 generated different levels of dsRNA depending on virus titres and cell types. Cells were treated with a series of viral dilutions of stock virus titres, between $10^{-1}$, the highest dilution, to $10^{-5}$, the lowest dilution (Appendix A). dsRNA production was quantified throughout fluorescent intensity and a varied intensity of dsRNA production was found. Instead of accumulating over time, it appears as though dsRNA production is cyclical, varying over time and titre (Figures 6, 7 & 8). Viral dsRNA was detected by J2, a monoclonal dsRNA-specific mouse antibody that specifically recognizes dsRNA of more than 40-bp length. dsRNA has a unique helical structure which provides an interactive surface for binding the antibody. dsRNA was visualized *in vitro* by immunofluorescence microscopy, with dsRNA stained green (FITC) and nuclei stained blue (DAPI). From the increasing virus dilutions experimental approach, the optimal virus dilution was determined for the time course experiments. Time points were chosen in which a peak signal for dsRNA was detected. To determine IF intensity, dsRNA amount in the cells was measured using Nikon NIS-Elements software to investigate the possibility of quantifying IF. Immunofluorescence strength differed between viruses (Appendix A). As expected, uninfected control cells were found to have less IF intensity, and other infected cells had

increased, but IF intensity without a predictable pattern with time and titre (Figures 4 & 5).

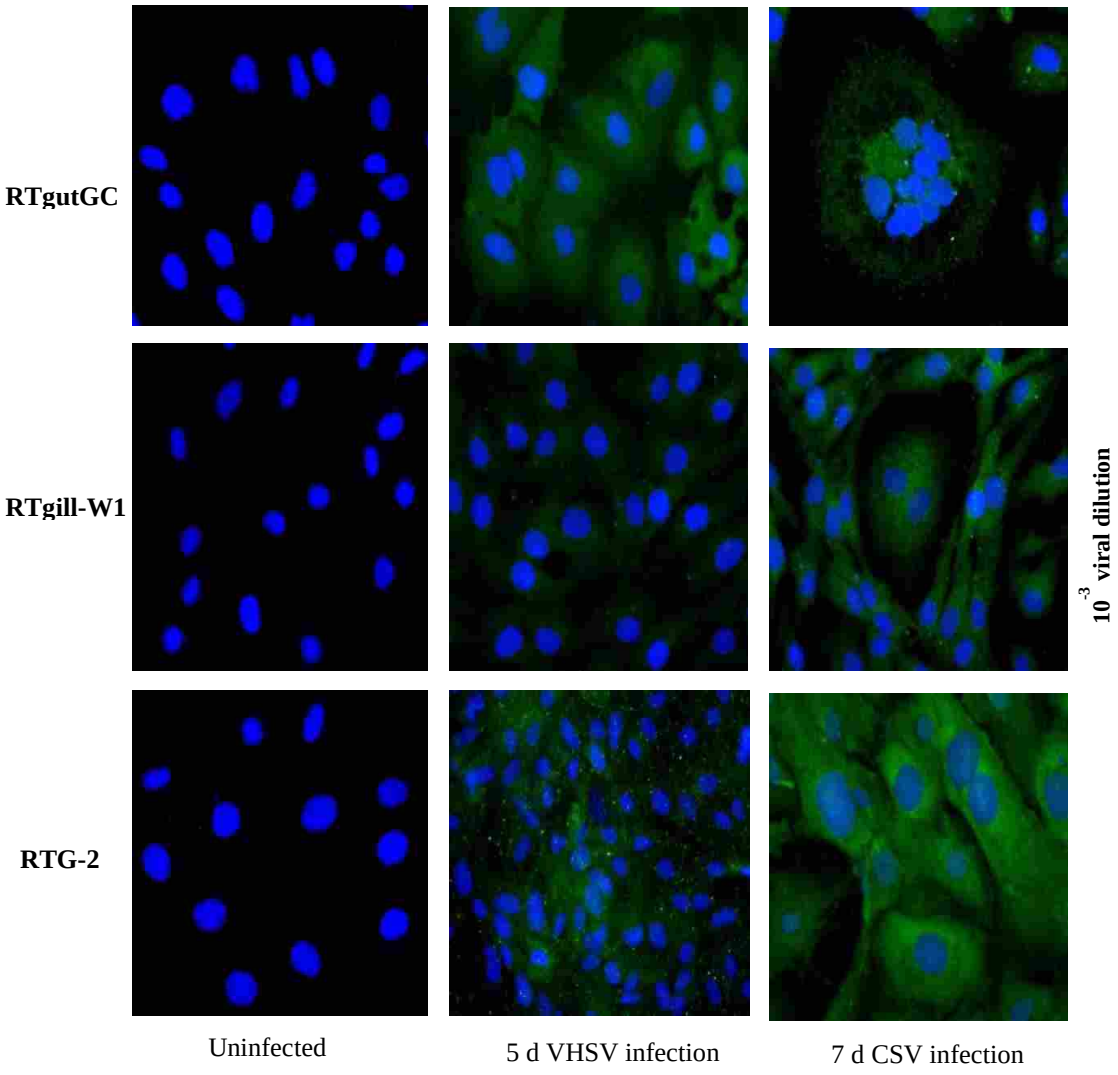


**Figure 4: dsRNA detection by immunofluorescence in VHSV and CSV infected fish cell lines.** RTgutGC, RTgill-W1 and RTG-2 cells were infected with $10^{-3}$ dilution of VHSV (stock = $10^5$ $TCID_{50}$/ml) and CSV (stock = $10^4$ $TCID_{50}$/ml) for 5 days with VHSV and 7 days with CSV; control cells were treated similarly in media without virus. dsRNA was detected by IF. The blue colour (DAPI) shows the cell nuclei and the green staining indicates viral dsRNA. All pictures were taken with the same magnification (400X).

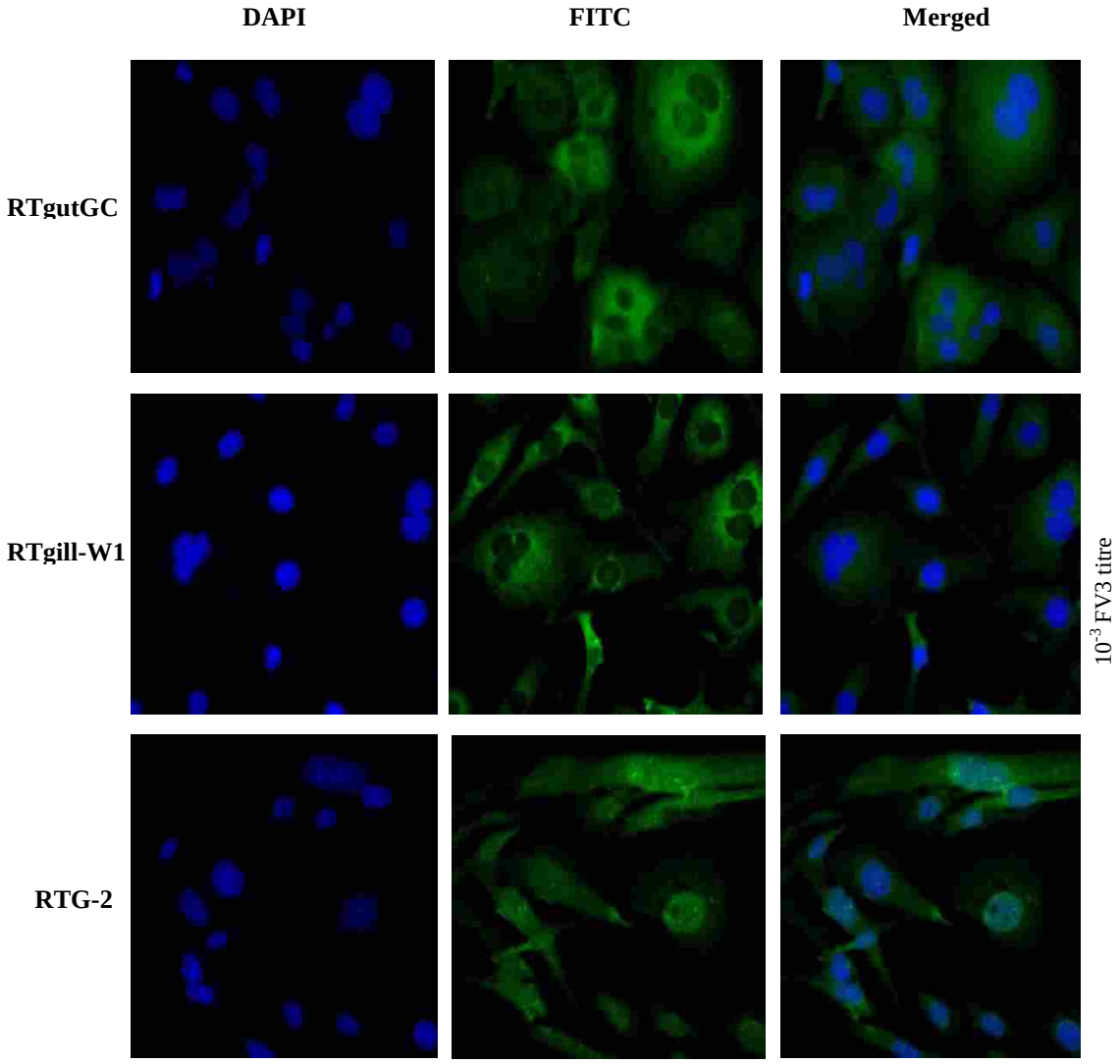


**Figure 5: dsRNA detection by immunofluorescence in FV3 infected fish cell lines**. Cells were infected with $10^{-3}$ dilution of FV3 (stock = $10^{6}$ $TCID_{50}$ /ml) for 2 days. dsRNA was detected by IF. The blue stain (DAPI) shows cell nuclei and the green staining indicates viral dsRNA. Within RTG-2, dsRNA molecules could be detected in the cytoplasmic and nuclear compartments; however, dsRNA molecules were detected in the cytoplasm alone in infected RTgutGC and RTgill-W1 cells. All pictures were taken with the same magnification (400X).

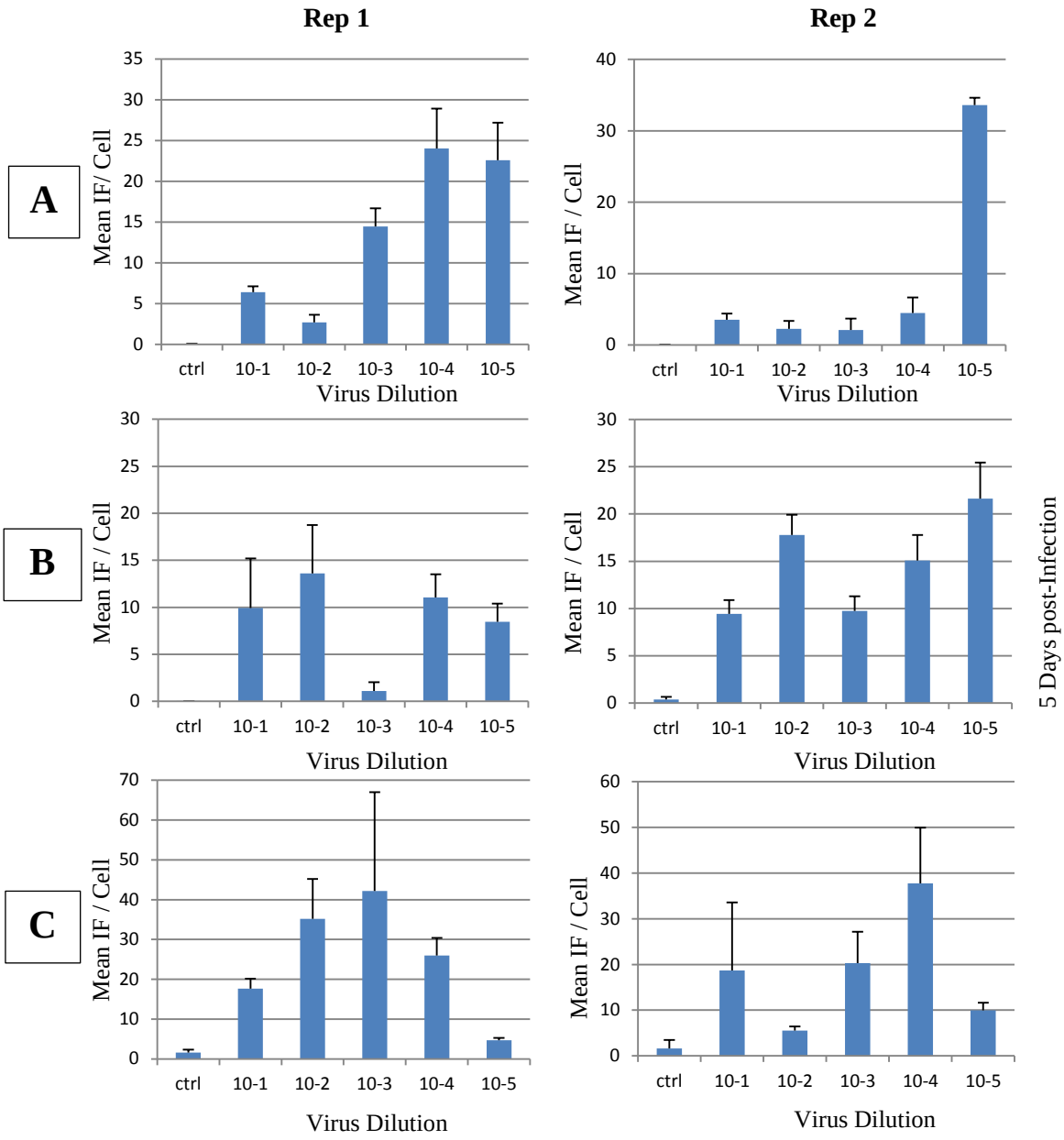


**Figure 6: Cyclical dsRNA production in rainbow trout cell lines infected with decreasing VHSV dilutions.** dsRNA production was measured using NIS-ELEMENTS BR software. Two replicates were performed for each cell line; dsRNA production is indicated as mean immunofluorescence (IF) on the y-axis, and the error bars indicate the average fluorescence/cell calculated from 3-4 images for each virus dilution. dsRNA production was cyclical within all the three rainbow trout cell lines: RTgutGC (A), RTgill-W1 (B), and RTG-2 (C).

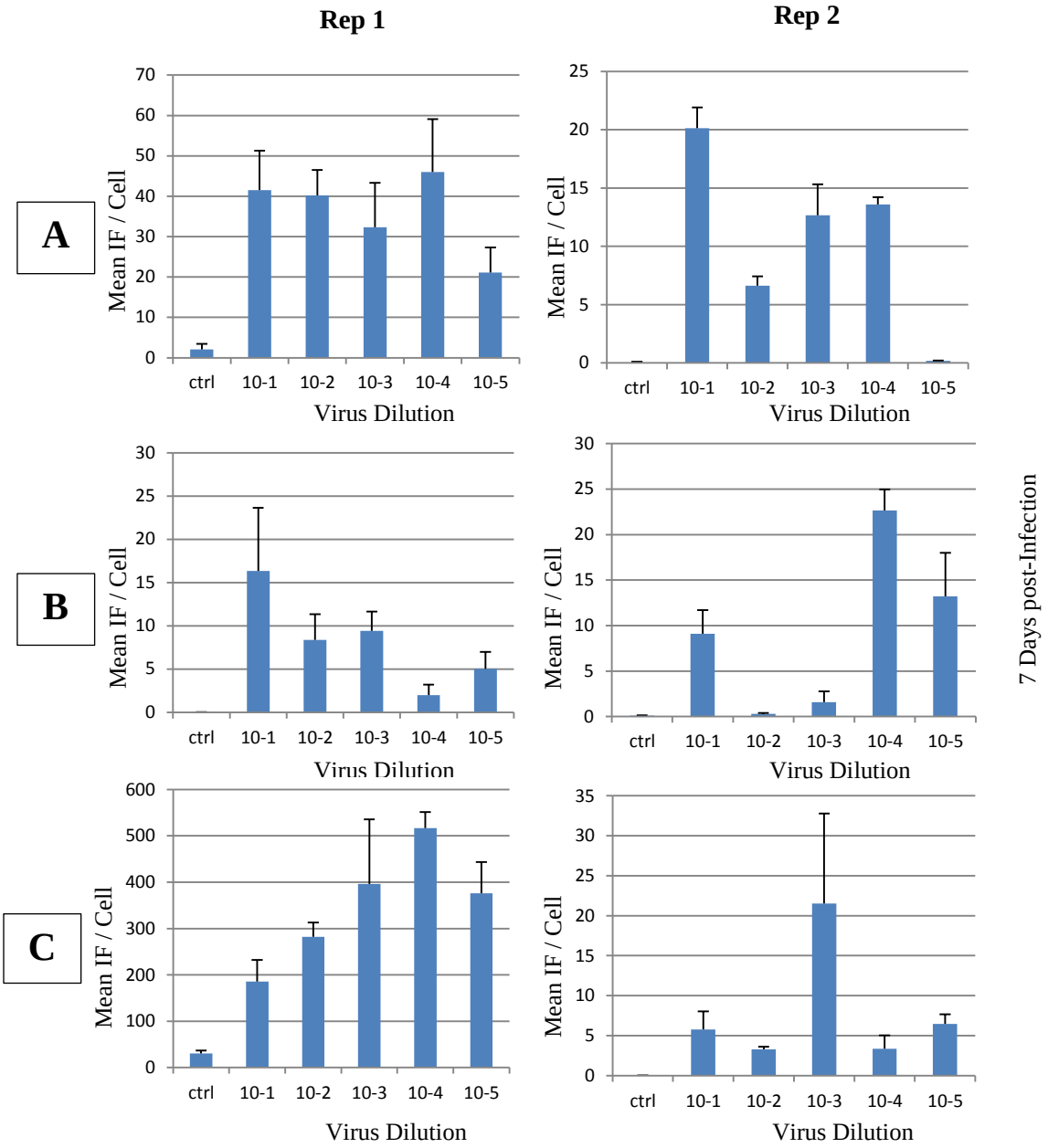


**Figure 7: Cyclical dsRNA production in rainbow cell lines infected with decreasing CSV dilutions.** dsRNA production was measured using NIS-ELEMENTS BR software. Two replicates were performed for each cell line; dsRNA production is indicated as mean immunofluorescence (IF) on the y-axis, and the error bars indicate the average fluorescence/cell calculated from 3-4 images for each virus dilution. dsRNA production was cyclical within all the three rainbow trout cell lines: RTgutGC (A), RTgill-W1 (B), and RTG-2 (C).

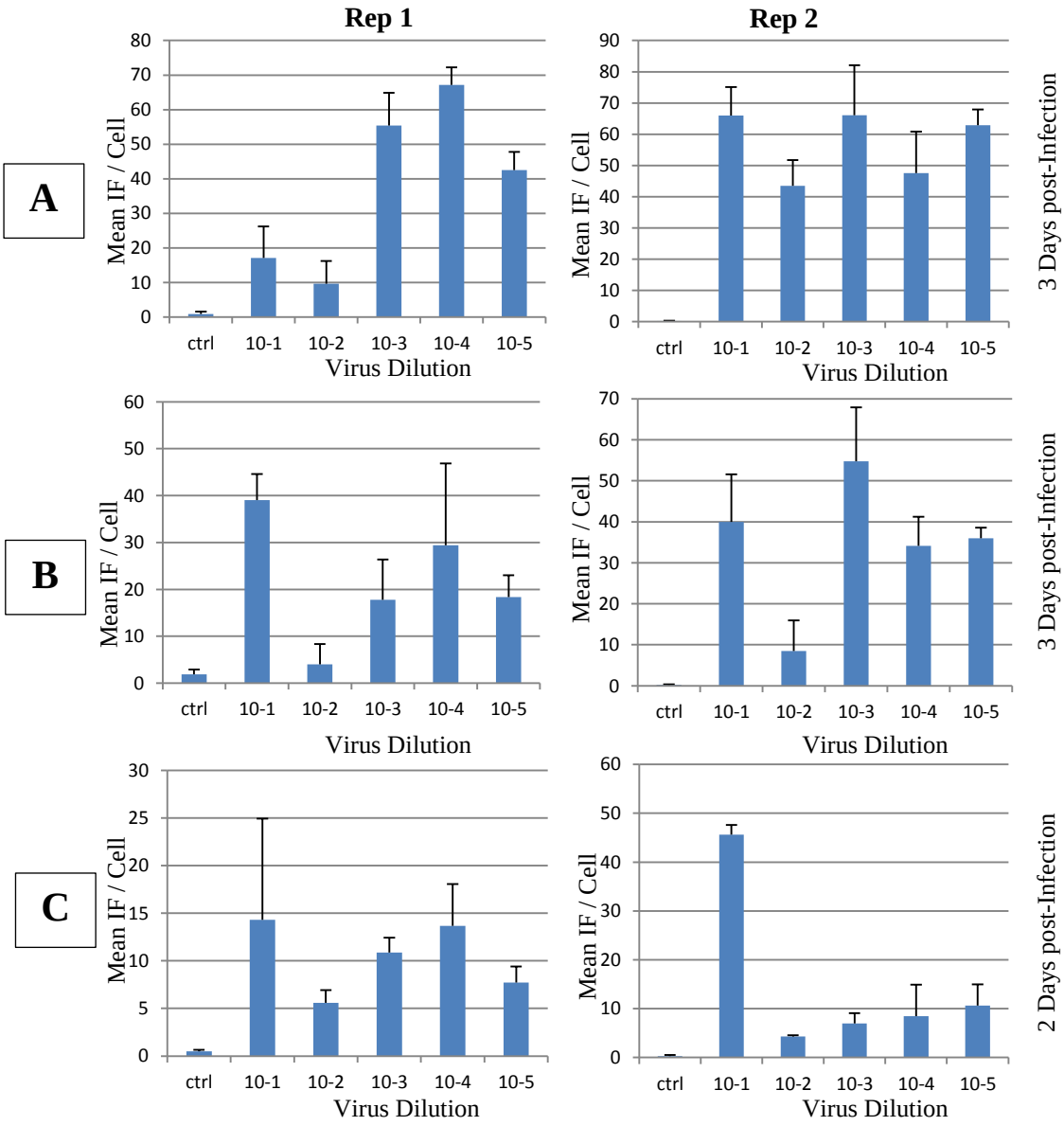


**Figure 8: Cyclical dsRNA production in rainbow cell lines infected with decreasing FV3 dilutions.** dsRNA production was measured using NIS-ELEMENTS BR software. Two replicates were performed for each cell line; dsRNA production is indicated as mean immunofluorescence (IF) on the y-axis, and the error bars indicate the average fluorescence/cell calculated from 3-4 images for each virus dilution. dsRNA production was cyclical within all the three rainbow trout cell lines: RTgutGC (A), RTgill-W1 (B), and RTG-2 (C).

### 4.1.2. Viral dsRNA production over time in fish cells using immunofluorescence

Time course experiments were performed to ascertain the day of peak dsRNA production. Based on the increasing viral titre experiments, the optimal virus dilution that produced the maximum amount of dsRNA was determined for each virus that had been used. Cells were infected with a viral dilution of $10^{-3}$ for VHSV and FV3 and for a $10^{-4}$ dilution of CSV (Figure 9). With VHSV, we have observed the production of dsRNA over one to five days with all rainbow trout cell lines: RTgutGC, RTgill-W1, and RTG-2. CSV also produced dsRNA during the entire incubation period, one day to seven days. While dsRNA production fluctuated between high and low, it was made by fish viruses at all virus dilutions and all time points with three rainbow trout cells. dsRNA production by fish viruses was measured using NIS-ELEMENTS BR software (Figures 10, 11& 12). It was observed that viral dsRNA signal appeared to peak and then go down; these fluctuations were observed both with varying times and viral dilutions.

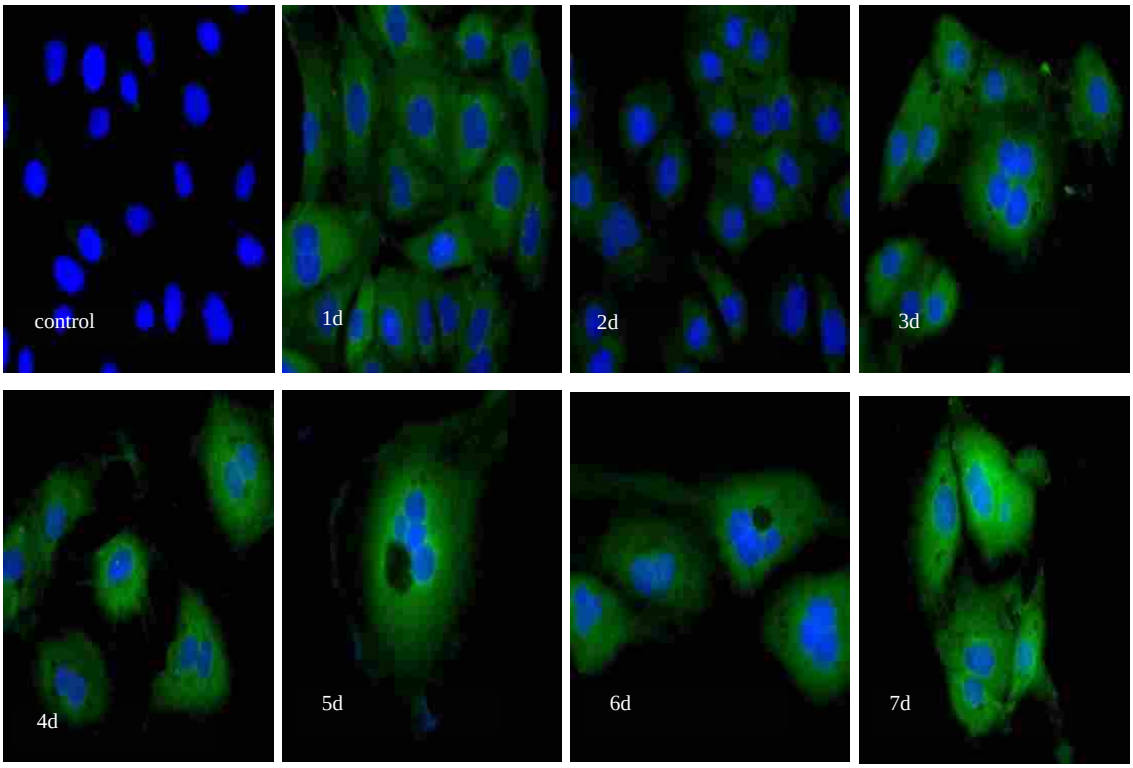


**Figure 9: Representative dsRNA production over time in RTgutGC infected with CSV.** The RTgutGC cell line was infected with $10^4$ $TCID_{50}$ /ml CSV for 1-7 days, and dsRNA production was detected using immunofluorescence microscopy. The blue stain (DAPI) indicates the cell nuclei and the green stain indicates viral dsRNA. Pictures were taken at the same magnification (400X).

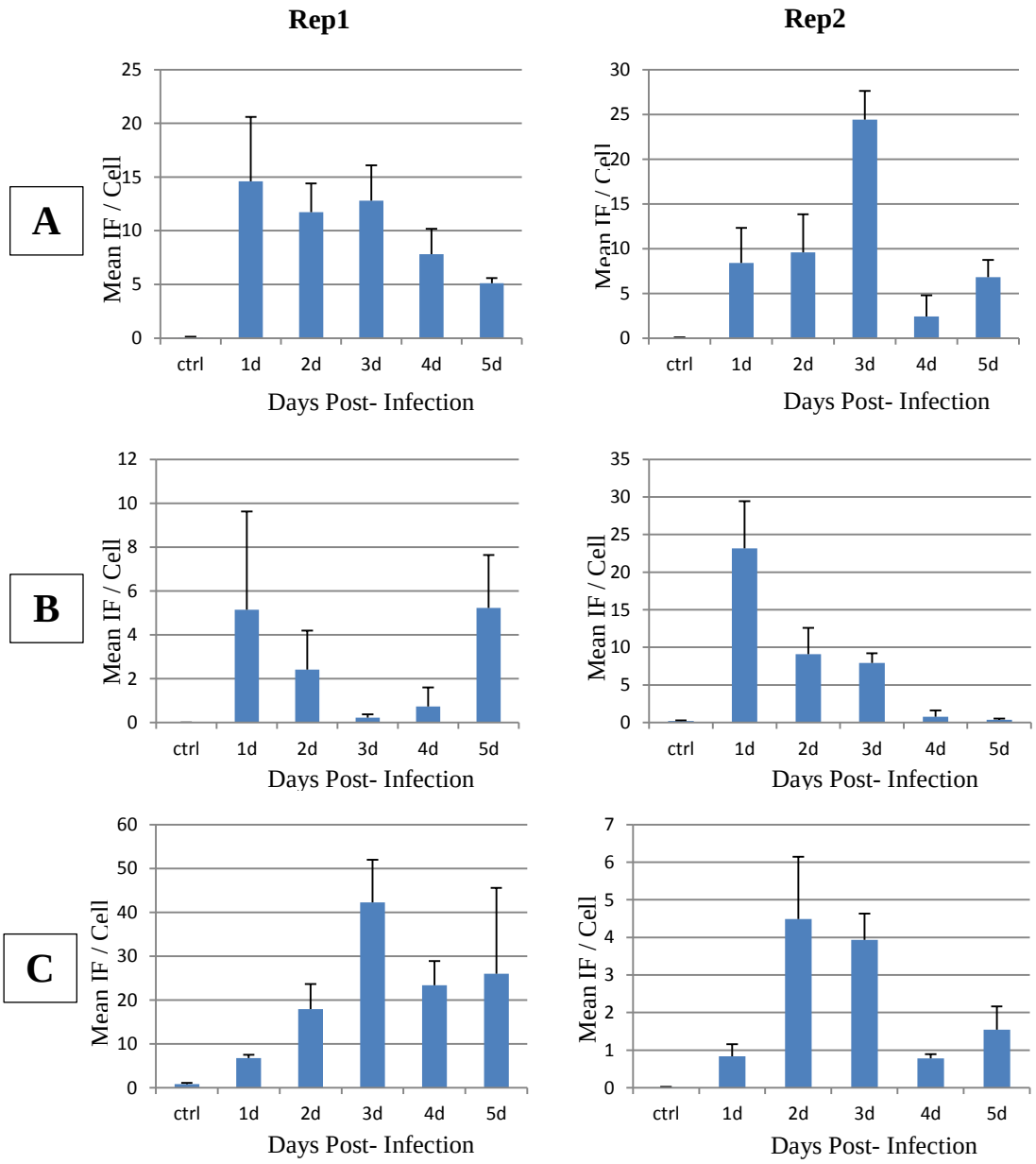


**Figure 10: Cyclical dsRNA production in rainbow cell lines infected with VHSV ($10^{-3}$ dilution) for varying time points.** dsRNA production was measured using NIS-ELEMENTS BR software. Two replicates were performed for each cell line; dsRNA production is indicated as mean immunofluorescence (IF) on the y-axis, and the error bars indicate the average fluorescence/cell calculated from 3-4 images for each virus dilution. dsRNA production was cyclical within all the three rainbow trout cell lines: RTgutGC (A), RTgill-W1 (B), and RTG-2 (C).

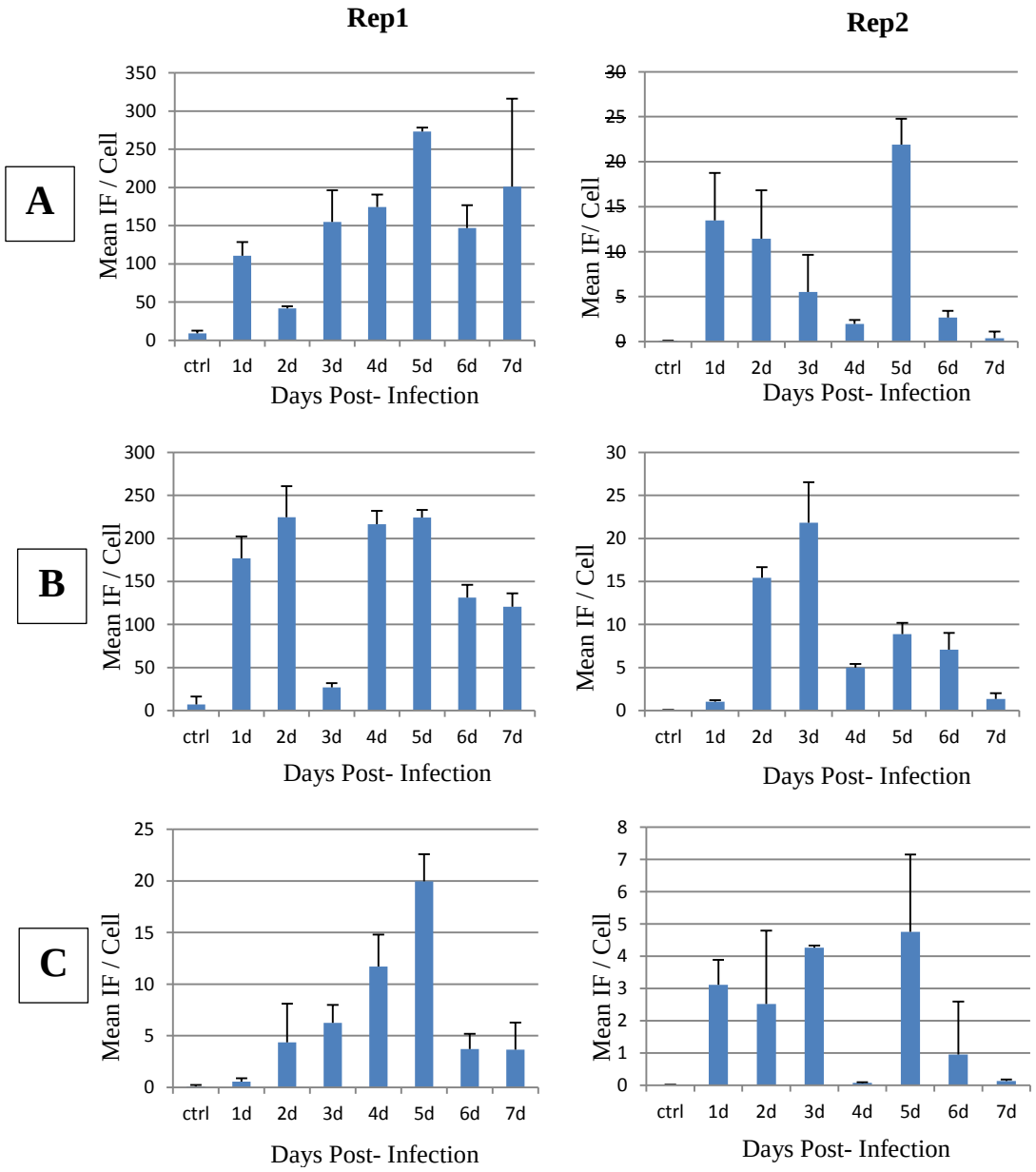


**Figure 11: Cyclical dsRNA production in rainbow cell lines infected with CSV ($10^{-4}$ dilution) for varying time points.** dsRNA production was measured using NIS-ELEMENTS BR software. Two replicates were performed for each cell line; dsRNA production is indicated as mean immunofluorescence (IF) on the y-axis, and the error bars indicate the average fluorescence/cell calculated from 3-4 images for each virus dilution. dsRNA production was cyclical within all the three rainbow trout cell lines: RTgutGC (A), RTgill-W1 (B), and RTG-2 (C).

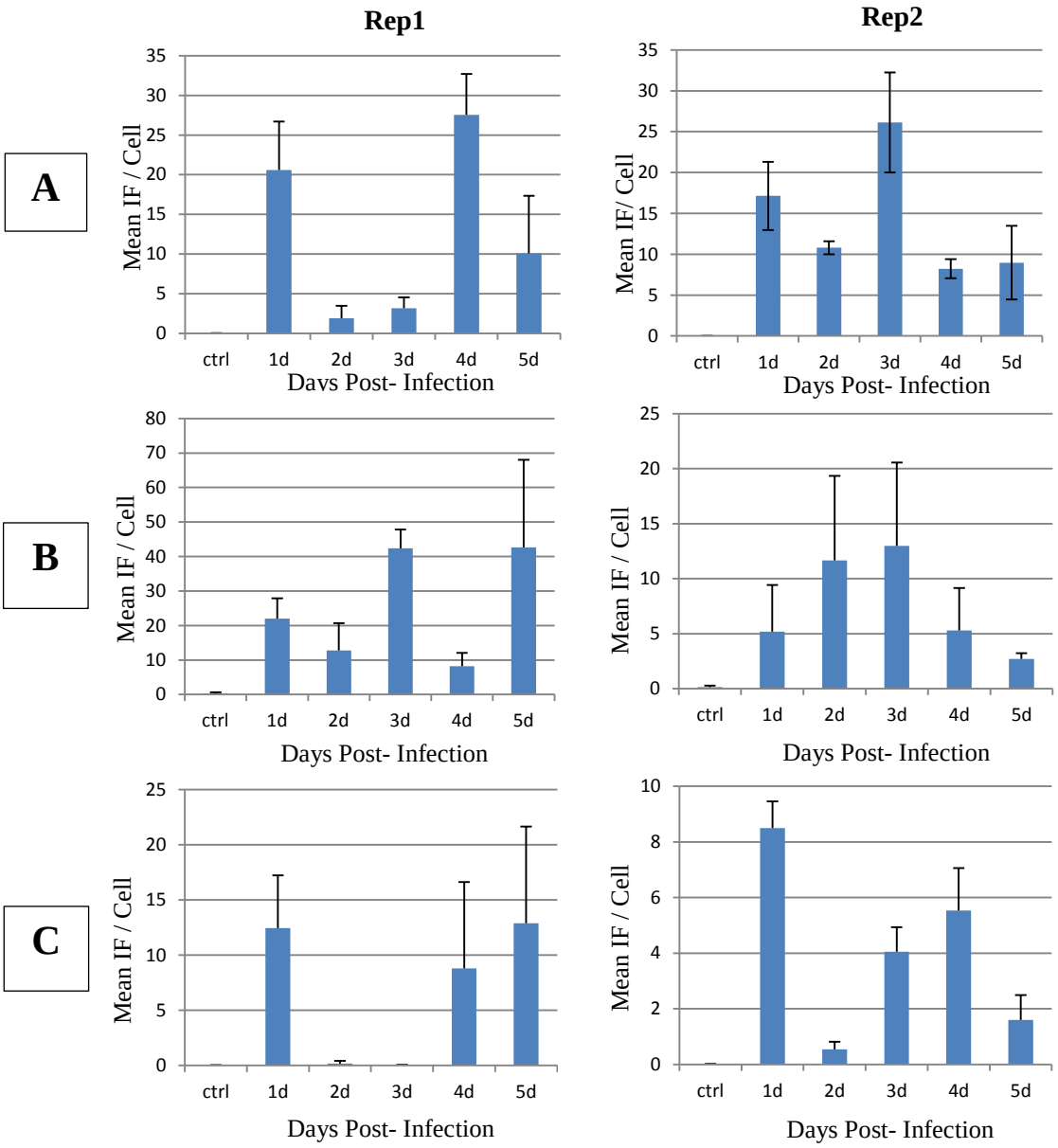


**Figure 12: Cyclical dsRNA production in rainbow cell lines infected with FV3 ($10^{-3}$ dilution) for varying time points.** dsRNA production was measured using NIS-ELEMENTS BR software. Two replicates were performed for each cell line; dsRNA production is indicated as mean immunofluorescence (IF) on the y-axis, and the error bars indicate the average fluorescence/cell calculated from 3-4 images for each virus dilution. dsRNA production was cyclical within all the three rainbow trout cell lines: RTgutGC (A), RTgill-W1 (B), and RTG-2 (C).

## 4.2. Characterizing viral dsRNA length, amount, and location

### 4.2.1. Determining location of viral dsRNA production within the cells

In the present study, the location of dsRNA could be determined for both RNA and DNA fish viruses using IF. It was determined that two RNA viruses, with double-stranded and negative-sense single stranded genomes, could produce dsRNA in the cytoplasm; while the DNA virus used in this study produced dsRNA in the nuclei and cytoplasm (Table 3). We observed dsRNA as a green stain surrounding the nuclei in the case of RNA virus infection which appeared to be in the cytoplasmic compartment (Figure 4); however, the green stain was both around and within the nuclei in the case of DNA virus infection with fibroblastic cells (RTG-2), and was only in the cytoplasm with epithelial cells (RTgutGC and RTgill-W1) (Figure 5).

**Table 3: Location of dsRNA production in fish cells.** VHSV and CSV produced dsRNA in the cytoplasm for all three cell lines tested. This correlates with the location where these viruses replicate their respective genomes; whereas, FV3 produced dsRNA in the cytoplasm for RTgutGC and Rtgill-W1 and in the nucleus and cytoplasm for RTG-2.

| | **Location of genome replication** | **RTgill-W1** | **RTgutGC** | **RTG-2** |
|---|---|---|---|---|
| **VHSV** | Cytoplasm | Cytoplasm | Cytoplasm | Cytoplasm |
| **CSV** | Cytoplasm | Cytoplasm | Cytoplasm | Cytoplasm |
| **FV3** | Nucleus<br>Nucleus/Cytoplasm | Cytoplasm | Cytoplasm | **Cytoplasm and Nucleus** |

### 4.2.2. Determining dsRNA length and amount using AO stain

To characterize the viral dsRNA produced by fish viruses in fish cell lines, we extracted total RNA from infected and uninfected fish cells, ran it on a 1% agarose gel, and then stained with acridine orange (AO). Both ssRNA and dsRNA are stained with AO, ssRNA is stained orange, and dsRNA is stained green (Figure 13). dsRNA was detected in infected cells but not in uninfected cells. In addition to determining the present of dsRNA, the AO gels were used to determine dsRNA length. Both VHSV and FV3 produced dsRNA with lengths ~ 20,000 bp (Figures 13 & 14). dsRNA length has not been measured for CSV.

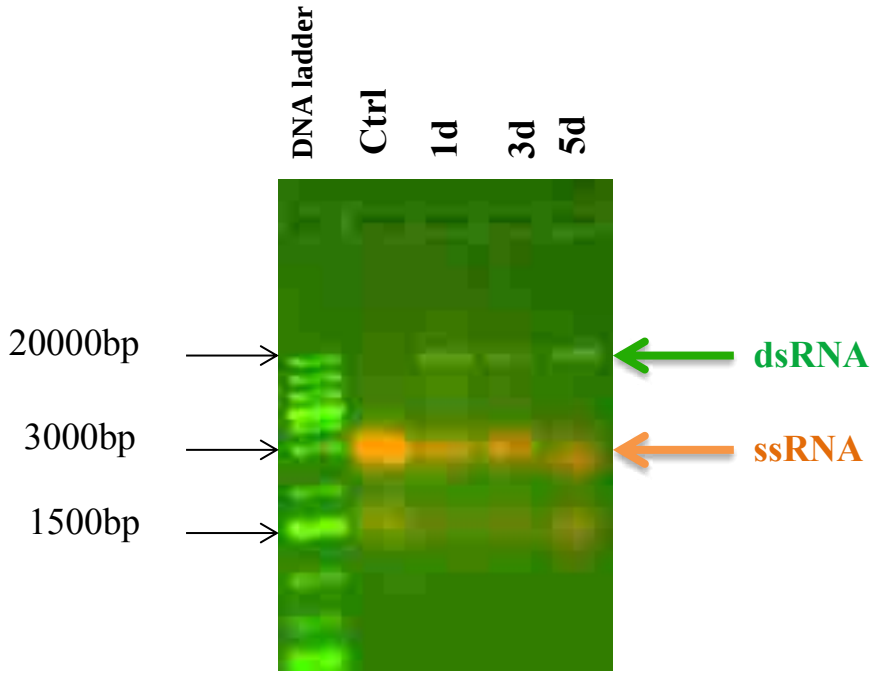


**Figure 13: dsRNA analysis by electrophoresis and acridine orange stained (AO) for VHSV infection:** RTgutGC cells were infected with $10^{-3}$ VHSV for one, three, and five days (time in days indicated across the top of the gel). dsRNA is stained green and ssRNA is stained orange. A dsDNA ladder is included for size approximation.

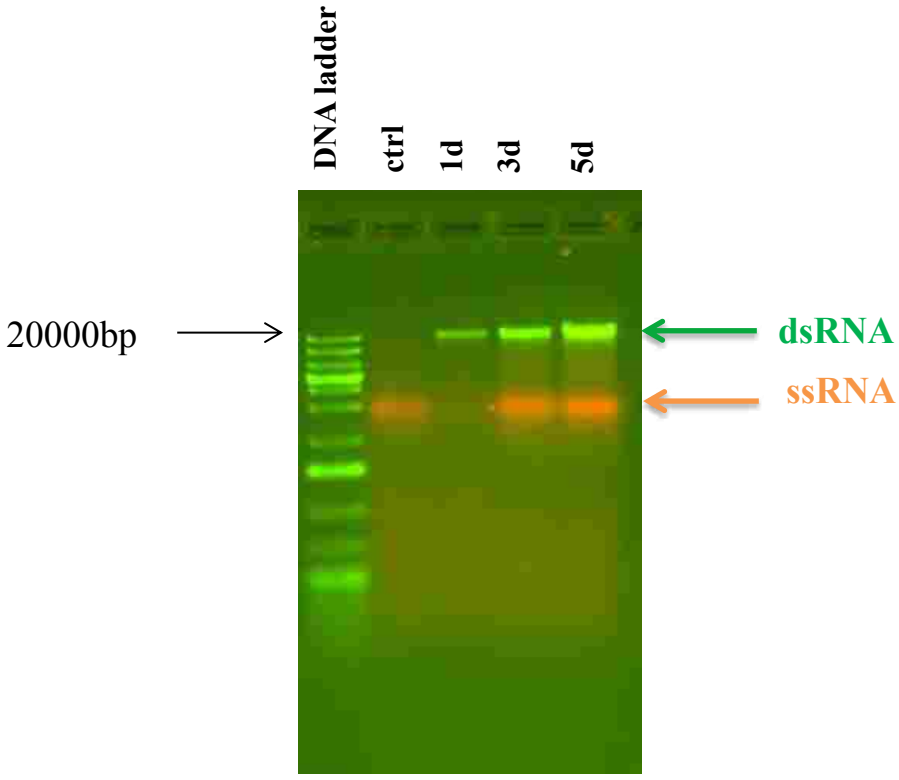


**Figure 14: dsRNA analysis by electrophoresis and acridine orange stained (AO) for FV3 infection:** RTgutGC cells were infected with $10^{-3}$ VHSV for one, three, and five days (time in days indicated across the top of the gel). dsRNA is stained green and ssRNA is stained orange. A dsDNA ladder is included for size approximation.

# 5. DISCUSSION

## 5.1. dsRNA is produced by all three fish viruses

In this study we aimed to test whether fish viruses have the ability to produce long dsRNA in fish cells. Most mammalian viruses generate dsRNA during their replicative cycle, as documented in mammalian cells (Table 2) (Jacobs & Langland, 1996; DeWitte-Orr & Mossman, 2010). Fish viruses, in contrast to other mammalian viruses, have not been tested for dsRNA production, even though they have induced innate antiviral immune responses (Table 1) and they possess dsRNA sensors and dsRNA-induced immune genes.

Three fish viruses - VHSV, CSV, and FV3 - were chosen to be tested in this study. These fish viruses were used for the goal of investigating the ability of dsRNA production by fish viruses with different replication cycles in fish cells. The data shows that all three fish viruses, with different genomes and thus different replication cycles, produced dsRNA in the three fish cell lines tested, both with increasing virus titres and time points. To our knowledge, this is the first study to show that fish viruses produce dsRNA in fish cell lines. Our results support the hypothesis that dsRNA is a natural feature of all viruses (Jacob, 1996; Kumar, 1998).

### 5.1.1. dsRNA production with increasing viral titres

$TCID_{50}$ (tissue culture infectious dose), is a common method used to measure a viral titre by estimating at what dilution of a viral stock results in fifty percent of a certain

endpoint. An endpoint in a titration of viruses is usually the dilution at which 50% of test animals or in this case a cell monolayer, dies (Reed & Muench, 1988). The $TCID_{50}$ for the viruses used in this study were determined to be: $10^5$/ml for VHSV, $10^4$/ml for CSV, and $10^6$/ml for FV3. Cells were exposed to a series of dilutions of the virus stock from $10^{-1}$ to $10^{-5}$. It was observed that viruses were able to produce dsRNA at all virus dilutions tested. Slight variations were found between dilutions, and this will be discussed further below.

This study showed that VHSV, a negative-sense ssRNA virus, is capable of producing dsRNA after 1-5 days of infection in all cell lines tested (Figure 4). This result correlates with Kato *et al.*'s (2008) study that showed that vesicular stomatitis virus (VSV), a mammalian negative-sense ssRNA virus, is able to generate dsRNA in mouse embryonic fibroblast (MEFs) cells. Nevertheless, this result is in disagreement with previous reports (Weber *et al.*, 2006) that showed that dsRNA is not produced by negative-sense ssRNA viruses. As the Weber *et al.* study did not investigate VSV or VHSV, their sweeping generalization regarding negative-sense ssRNA viruses is not true, and perhaps a small subset of viruses with this genome are unable to produce dsRNA.

CSV is able to form long dsRNA after 1-7 days post infection in all three cell lines tested (Figure 4). We expected that CSV would produce dsRNA since it has a dsRNA genome. According to Weber *et al.*, dsRNA virus infections result in the production of significant amounts of dsRNA (Weber *et al.*, 2006). In case of dsRNA viruses, we know that some of the genomic dsRNA produced must becoming detectable by the cell (ie. released from capsids during virus replication cycle), as ISGs are induced during a CSV infection in rainbow trout cell lines (DeWitte-Orr *et al.*, 2007). Thus, the

dsRNA that was detected during a CSV infection definitely would be genomic, but there is likely also genomic dsRNA escaping the capsid and acting as PAMPs to stimulate an antiviral response (DeWitte-Orr *et al.,* 2007). Furthermore, it is important to note that CSV, with its dsRNA genome, can act as a positive control for the dsRNA production experiments in this study.

In the current study, FV3 was able to produce dsRNA in fish cells (Figure 5). FV3 is a highly potent virus that kills cells quickly; furthermore, apoptosis has been induced by FV3 in fathead minnow (FHM) cells and baby hamster kidney (BHK) cells between 3 to 17 hrs (Chinchar *et al.,* 2003). Thus, cells were treated with dilutions of FV3 for much shorter time points compared with VHSV and CSV (1-3 days with RTgill-W1 and RTgutGC, and 1-2 days with RTG-2).

It is valuable to note that all three viruses produced dsRNA in all three cell lines, even when diluted $10^{-5}$. This suggests that all three viruses are capable of producing copious amounts of dsRNA, and as such, this dsRNA is easily detected by the J2 antibody used in this study for IF.

### 5.1.2. dsRNA production over time

Not only is it important to determine the optimal virus dilution for producing dsRNA, but also the optimal time point. Thus time course experiments were performed to investigate the best time for virus dsRNA production by VHSV, CSV and FV3 in the three cell lines tested.

It was determined that neither the highest viral dilution nor the lowest were optimal titres for inducing dsRNA. It is unclear why this trend was observed, perhaps $10^{-1}$ dilutions caused the cells to become overwhelmed and unable to support a productive infection, while $10^{-5}$ dilutions did not have sufficient virus numbers to support optimal dsRNA production. The virus dilutions that supported optimal dsRNA production were the middle viral dilutions ($10^{-3}$ and $10^{-4}$), and as such these dilutions were chosen for the time course experiments. The optimal dilution for each virus used in this study was determined as follows: $10^{-4}$ viral dilution for CSV, and $10^{-3}$ viral dilution for VHSV and FV3. Viral dsRNA production was evaluated on days 1, 2, 3, 4, and 5 post-infection with VHSV, while viral dsRNA production was observed until 7 days post-infection with CSV (Figure 4). Interestingly, DNA virus (FV3) had two optimal times depending on cell type: 3 days incubation was best time for epithelial cells (RTgill-W1 and RTgutGC), while 2 days incubation was best for fibroblastic cells (RTG-2). This indicates that FV3 produces dsRNA faster in RTG-2 than RTgill-W1 and RTgutGC. This suggests that RTG-2 may not be able to defend itself as well against FV3 compared with the epithelial cell lines tested.

It is important to note that all three viruses were able to produce dsRNA in all three cell lines within 1 day of infection. This suggests that these viruses are replicating quickly within the cell lines tested and that dsRNA is produced early on in an infection.

## 5.2. Epithelial and fibroblastic rainbow trout cell lines support viral dsRNA production and virus replication

In this study, three fish cell lines were used to detect dsRNA production by fish viruses. The cell lines were: 1) RTgutGC, 2) RTgill-W1, and 3) RTG-2. All three cell lines supported the production of dsRNA by the three viruses tested. There did not appear to be any observable differences in dsRNA production (ie. ability to produce dsRNA, its location within the cell, pattern of cytoplasmic staining) between the three cell lines with respect to CSV and VHSV infections. Differences were observed between the three cell lines with respect to FV3 infections, this will be discussed later in the discussion.

It is important to note that all the experiments performed to monitor dsRNA production were performed using sub-lethal conditions. At no time during these experiments were the cells dying. However, if these experiments were performed for longer lengths of time, classic cytopathic effect (CPE) was observed for the three viruses. VHSV and FV3 caused cell death in all three cell lines tested. FV3 killed RTG-2 more quickly than RTgutGC and RTgill-W1. This and other observed differences between the cell lines with FV3 are currently being investigated. CSV was found to produce its classic CPE, syncytia formation in all cell lines tested. Syncytia formation, the fusion of cells to form a multi-nucleated giant cell, has been shown previously in fish cells at 7 days with fibroblastic cell lines and 4 days with epithelial cell lines (DeWitte-Orr & Bols, 2007). In the current study, CSV was also observed to induce syncytia formation but at later time points (Figure 9) (Appendix B). The delayed syncytia formation observed in this study is likely due to lower virus titres used compared with the previous study.

## 5.3. dsRNA production patterns ascertained using IF

dsRNA production by IF was quantified using Nikon NIS-ElEMENTS software, where fluorescence intensity/cell was hypothesized to correlate with dsRNA quantity. As such, we noted that the production levels of dsRNA during a time course or virus dilution experiment tended to reached a peak in fluorescence and then decrease in fluorescence intensity. This obervation was contrary to what was expected, where dsRNA would accumulate in the cell until the cell lysed. We expected dsRNA to accumulate within the cell because dsRNA is a stable molecule and is generally nuclease resistant (DeWitte-Orr & Mossman, 2010). However this was not the case, and the results suggest a cyclical production of dsRNA. There are at least three possible explanations for the results observed. Firstly, the infections may not be synchronous between treatments and this could explain differences in dsRNA observed. This is unlikely; however, because synchronized virus infections (infections performed at 4$^{o}$C and brought up to room temperature) did not show the accumulation of dsRNA, but were cyclical as well (data not shown). Secondly, dsRNA could be somehow released from the cell during the infection using a mechanism other than cell lysis. All the time points and virus dilutions used in this study were sub-lethal, therefore the dsRNA would not be leaving the cell by lysis. To our knowledge there is no known mechanism for dsRNA to be released from the cell other than cell lysis, and this would be a very interesting phenomenon to pursue in the future. Finally, it is possible that the dsRNA is being effectively degraded in fish cell. Previous studies have suggested that mammalian cells do not appear to be efficient at degrading dsRNA (DeWitte-Orr *et al.,* 2009). It is possible that fish cells express

dsRNases that are more efficient or expressed at higher levels that those in mammals. This would also be an interested hypothesis to pursue in future studies.

## 5.4. Location of dsRNA production associated with virus replication location

Microscopy was used to observe the location of dsRNA production within the cell. VHSV and CSV replicate their genomes in the cytoplasmic compartment, and thus it was hypothesized that these viruses would generate dsRNA in that location as well. This hypothesis was determined to be true, we found that both VHSV and CSV produced dsRNA in the cytoplasm of all three rainbow trout cell lines tested (Figure 4). FV3 replicates its genome in two steps or stages, first in the nucleus and then secondly within the cytoplasm of the cell (Kumar & Carmichae, 1998). Interesting, the data shows that FV3 produced dsRNA in both the cytoplasm and nuclear compartments in RTG-2 while, but it produced dsRNA only in the cytoplasm in RTgutGC and RTgill-W1 (Figure 5). This is the only significant difference in the location of dsRNA production that was observed between cell lines. This data suggests that FV3 replicates differently between the two cell types (fibroblasts and epithelial cells). This observation and other data generated in the lab (unrelated to this thesis) suggest that it is likely RTG-2 is more susceptible to FV3 infection compared with the epithelial cell lines. It is likely that the time points we have chosen miss the nuclear stage of virus replication in RTgutGC and RTgill-W1, and only the second, cytoplasmic stage is observed. RTG-2 which demonstrated both nuclear and cytoplasmic dsRNA suggests either the infection is delayed in this cell line, or the infection is somehow progressing differently. It is unlikely

the former explanation is the case, as we have observed that RTG-2 is killed faster than RTgutGC and RTgill-W1, suggesting the virus infection progresses faster in RTG-2. Data generated by another project in the lab has shown that RTG-2 is also unable to mount as effective antiviral response against FV3 compared with the epithelial cell lines. Thus the later explanation is more likely the case, whereby the FV3 infection in RTG-2 progresses differently compared with RTgill-W1 and RTgutGC. The mechanism of its replication within these three cell lines in currently under investigation.

## 5.5. dsRNA length and amount determined using an antibody-independent method

The antiviral response is dependent on dsRNA length with longer molecules inducing a stronger immune response; therefore, dsRNA length is significant for studying antiviral response at the cell culture level (DeWitte-Orr & Mossman, 2010; DeWitte-Orr *et al*., 2009; Kato *et al*., 2008). In the current study, double stranded RNA size was characterized by using an acridine orange (AO) stain assay. The AO assay was used to prove dsRNA production by the three fish viruses using an antibody-independent assay. AO is a metachromatic dye that stains double-stranded nucleic acids green and single-stranded nucleic acids red (McMaster & Carmichael, 1977). In RTgutGC infected with VHSV over a time course (1, 3, and 5 days), dsRNA was detected at all three time points. Uninfected cells did not show the presence of dsRNA. Infected and uninfected cells had two ssRNA bands, which represent 28S and 18S rRNA (Figure 13). VHSV produced long dsRNA molecules, approximately 20 kbp in length in fish cells (Figures 13). Because VHSV has a non-segmented genome, long dsRNA molecules were

expected to be generated from full-lengths of the genome self-annealing. The VHSV genome is 11kb in length; however the dsRNA on the gel appears to be closer to 20kbp. Thus, to determine the exact size of the dsRNA produced we suggest using a high molecular weight ladder and running the RNA on a gel with less than 1% agarose for longer periods of time to more accurately elucidate the size of the dsRNA produced.

dsRNA length was also determined from RTgutGC infected with FV3. The data shows that dsRNA was present in the virus-infected cells but not in healthy, uninfected cells. FV3 also appeared to produce dsRNA of approximately 20kbp in length. This was unexpected as FV3 has a fragmented genome (Chinchar *et al.*, 2011) and would likely produce dsRNA reflecting the length of the genomic fragments. It possible that the AO assay is not able to detect the smaller dsRNA fragments as they would overlap with the orange ssRNA bands on the gel. It is also possible that the RNA extraction method used (Trizol) supports the annealing of RNA, making very large dsRNA molecules from the smaller fragments. Degrading the ssRNA by selective nuclease degradation and using alternative RNA extraction methods would be recommended to finesse the AO gel assays for dsRNA detection.

In addition to demonstrating the presence and length of dsRNA in FV3 and VHSV infected cells, the AO gels provided two other interesting observations. Firstly, in with FV3, much of the ssRNA bands appeared to be missing (Figure 14). FV3 codes for at least three nucleases that are capable of degrading ssRNA (Kang & McAuslan, 1972). It is likely that these virus-associated nucleases are degrading cellular ssRNA molecules. This would advantageous to the virus, if host mRNA transcripts were degraded then virus transcripts would be preferentially translated by the host cell's machinery (Chinchar *et*

*al.,* 2003). It is also possible that FV3 is activating the host innate immune response, namely the OAS pathway. When dsRNA is produced it binds OAS, which oligomerizes and activates RNaseL. RNaseL then degrades ssRNA, blocking protein synthesis of both the host and virus (DeWitte-Orr & Mossman, 2010; Robertsen, 2006). Secondly, the AO gels did not show the cyclical effect of dsRNA production as determined in IF assay, and appeared to accumulate. There are differences in the two techniques in how they detect dsRNA. dsRNA detected by IF is on an individual cell basis while with AO assay, RNA was extracted from the complete culture, thus the AO gel is a snapshot of the culture as a whole Thus it can be concluded that on a per cell basis dsRNA accumulates in a cyclical fashion, but on the culture as a whole it appears to accumulate. Therefore in future studies when making conclusions regarding dsRNA accumulation it will be important to note the method of detection used. CSV was not tested for dsRNA using the AO stain since CSV is dsRNA genome and we know that it can produce a long dsRNA in length; however, could prove interesting in determining dsRNA length produced by dsRNA virus (CSV).

# 6. CONCLUSIONS

In conclusion, our work is the first to demonstrate that the RNA and DNA fish viruses VHSV, CSV, and FV3 produce dsRNA using two techniques: Ab-depended assay (IF) and Ab-independed assay (AO). Our data correlates with previous studies in mammals that demonstrate the formation of dsRNA which is thought to be a general feature of all viruses (Jacob & Langland, 1996; Kumar & Carmichael, 1998). Fish viruses were chosen in terms of genome types including negative sense-ssRNA, dsRNA, and dsDNA. The objective was to test how different virus with different genome types form dsRNA. During this process, we were also able to demonstrate the optimal time and titres for each virus. No great differences between VHSV and CSV were observed regarding the production of dsRNA in the three rainbow trout cells RTgutGC, RTgill-W1, and RTG-2 with both viruses producing dsRNA in the cytoplasm compartment in which their genomes replicate. However, dsRNA formation was observed until 7 days by CSV and 5 days by VHSV. FV3 proved to be a very interesting virus that replicates in a different way in RTG-2 compared to RTgutGC and RTgill-W1. Thus, FV3 had two optimal incubation times depending on the cell type: either 2 days (RTG-2, fibroblast) or 3 days (RTgutGC and RTgill-W1, epitheslial). Moreover, FV3 produced dsRNA in two different compartments in the nuclei and cytoplasm with RTG-2, but produced dsRNA only in the cytoplasm with RTgutGC and RTgill-W1. dsRNA production appeared to be cyclical at the individual cell level, which may suggest that dsRNA may be is being degraded or released from the cells during virus infection.

We studied rainbow trout cell lines in this study as a tool for examining antiviral responses (Lakra *et al.,* 2011). It is clear that rainbow trout and salmon are currently

threatened by VHSV and CSV. Due to acting as a carrier for FV3, rainbow trout and salmon could be threatened in the future by FV3. This study is the first to demonstrate that FV3 replicates and induces immune responses in rainbow trout. As well, this study is the first to study virus infections in RTgutGC and RTgill-W1. It was demonstrated that RTG-2, RTgill-W1, and RTgutGC are able to support virus replication; moreover, all three viruses produced plentiful dsRNA in these cell lines. IF was used to identify dsRNA in the cells using the J2 (anti-dsRNA) antibody. This method is commonly used to detect dsRNA in mammalian cells using fluorescence microscopy (Weber *et al.*, 2006). An acridine orange stain (AO) assay was used to determine the length of dsRNA produced by VHSV and FV3. Our data indicates that VHSV and FV3 produced long dsRNA ~ 20 kbp in length; nevertheless, more research and alternative techniques are needed to investigate how these two viruses with different genome types and sizes were able to produce dsRNA of similar size. As well, further AO studies are needed to measure dsRNA produced by CSV. Overall, our work not only proved the presence of dsRNA, but also determined the location and the length of dsRNA produced by fish viruses in fish cell lines.

# 7. FUTURE DIRECTIONS

Viral dsRNA is considered to be one of the most important pathogen-associated molecular pattern (PAMP), and is a potent inducer of type I IFN (DeWitte-Orr & Mossman, 2010). In this study, dsRNA production, location, and length were determined; however, it is clear that this research should be continued and expanded to continue to understand virus-host interactions in fish. For future directions, I suggest immunoblot assays should be performed. This assay is essentially a western blot there RNA is run on a gel instead of protein. Immunoblots would be an ideal method to measure dsRNA length using the J2 dsRNA antibody, and would confirm the size of dsRNA that was observed using the AO assay. The AO assay and an immunoblot differ in that the AO assay cannot differentiate between dsRNA and dsDNA and the ssRNA bands may be interfering with observing similar sized dsRNA bands; however, with an immunoblot only dsRNA is detected; therefore, the level of accuracy is increased. Moreover, the total RNA could be treated with RNase A to degrade ssRNA prior to being loaded onto the AO gel to insure that only dsRNA is present and ssRNA would not be able to mask smaller dsRNA bands. Furthermore, dsRNA production could be confirmed in virus-infected fish tissue by immunohistochemistry (IHC). IHC helps to visualize the distribution and localization of dsRNA inside a tissue, and can be performed using the J2 antibody. Moreover, although dsRNA production was observed by fish viruses since day one, we do not know the exact time point in which the virus starts to form dsRNA. Therefore, it would be valuable to try earlier time points such as 1, 3, 6, 12, and 24 hrs to identify the exact time point at which dsRNA is produced, and compare this with the time of viral genome replication. The time of virus replication detection could be achieved

using reverse transcription polymerase chain reaction (RT- PCR). Finally, differences in the innate immune response to FV3 in rainbow trout cell lines RTG-2, RTgutGC, and RTgill-W1 need to be investigated to understand how these cell lines defend against this virus, and how this virus replicates within this cell lines.

The long term objective of this study is to extract the dsRNA molecule produced by the virus and use it to treat naïve cells to investigate how native dsRNA induces an antiviral state in healthy cells and is able to control a virus infection. Therefore, cellular responses to dsRNA are valuable when studying viral pathogenesis. In the future, this study could contribute to novel methods of protecting fish populations from serious viral diseases. Such protection could eventually cause growth in the aquaculture industry both globally and especially in Canada. Finally, innate immune responses are conserved between animals. Thus findings in fish could also contribute to the understanding of innate immune responses in humans, which could improve and enhance human health.

# 8. INTEGRATIVE RESEARCH APPROACH

Science is at its best and most productive when working within an integrative environment. This study used fish cell lines and viruses and focused on how fish cells respond to viral infection and produce viral dsRNA in the host infected cells. The integrative nature of this project allows for the combination of diverse methodologies to achieve its research objectives. These methods include: cell culture, virus culture, immunofluorescence (IF), and acridine orange stain (AO) methods. Each method functions uniquely in helping to integrate data and statistics from different angles and experiences to better understand and reach my research objectives.

This research also bridges biology disciplines using a multidisciplinary approach to solve problems. The fields of virology, immunology, molecular biology, cellular biology, and health science were all applied to this research of identifying the presence of viral dsRNA molecules, characterizing the viral dsRNA molecule including its length and localization within the cell, addressing the interaction between different fish cell types with various fish viruses, and studying viral pathogenesis. In summary, the present study is truly integrative in its use of many methods and biological disciplines to provide an overview of the response of the fish cells to viral infection.

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# 10. APPENDIX

**Appendix A. The following figures represent one of the two IF experiments performed to determine dsRNA production in the three cell lines with three fish viruses.**

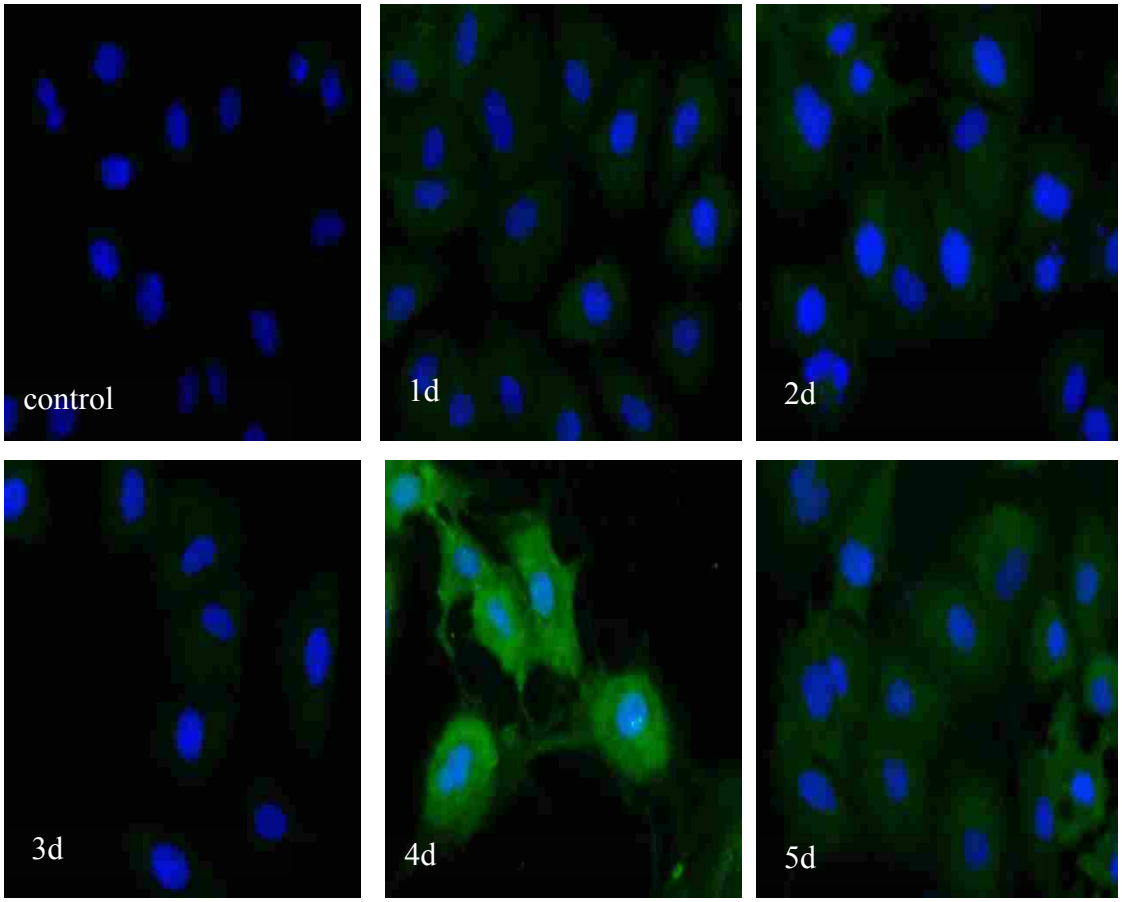


**Figure A-1: DsRNA production as determined by IF in RTgutGC infected with $10^{-3}$ VHSV time course (1 – 5 days).**

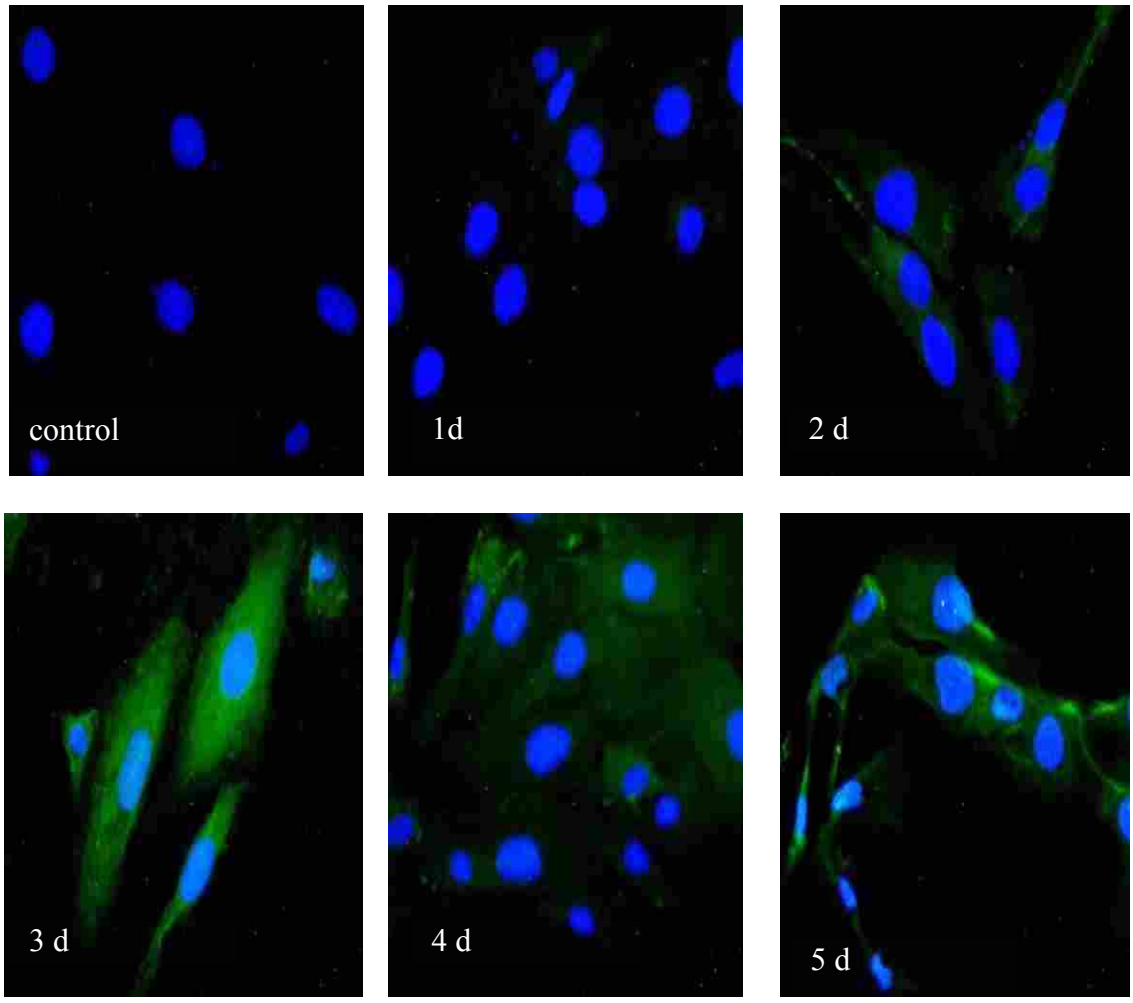


**Figure A-2: DsRNA production as determined by IF in RTG-2 infected with $10^{-3}$ VHSV time course (1- 5 days).**

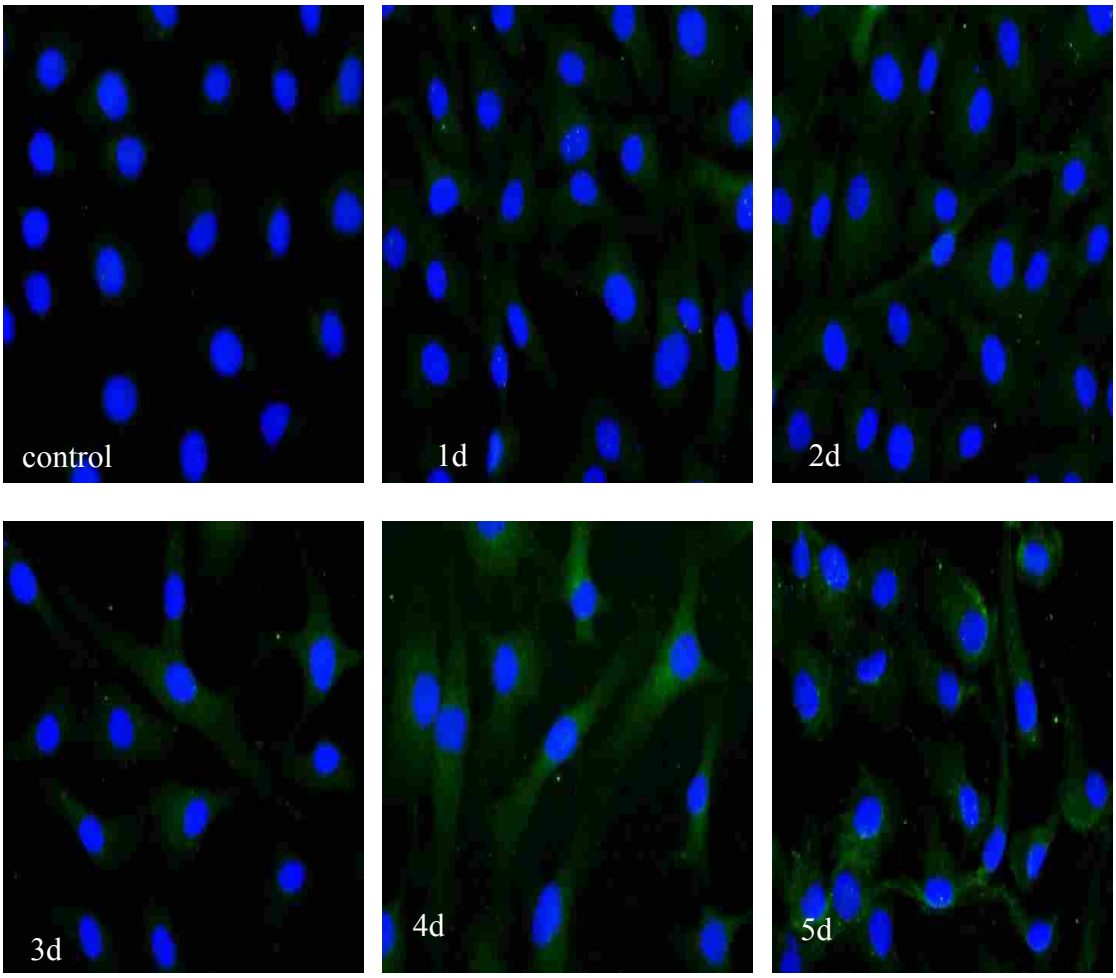


**Figure A-3: DsRNA production as determined by IF in RTgill-W1 infected with $10^{-3}$ VHSV time course (1-5 days).**

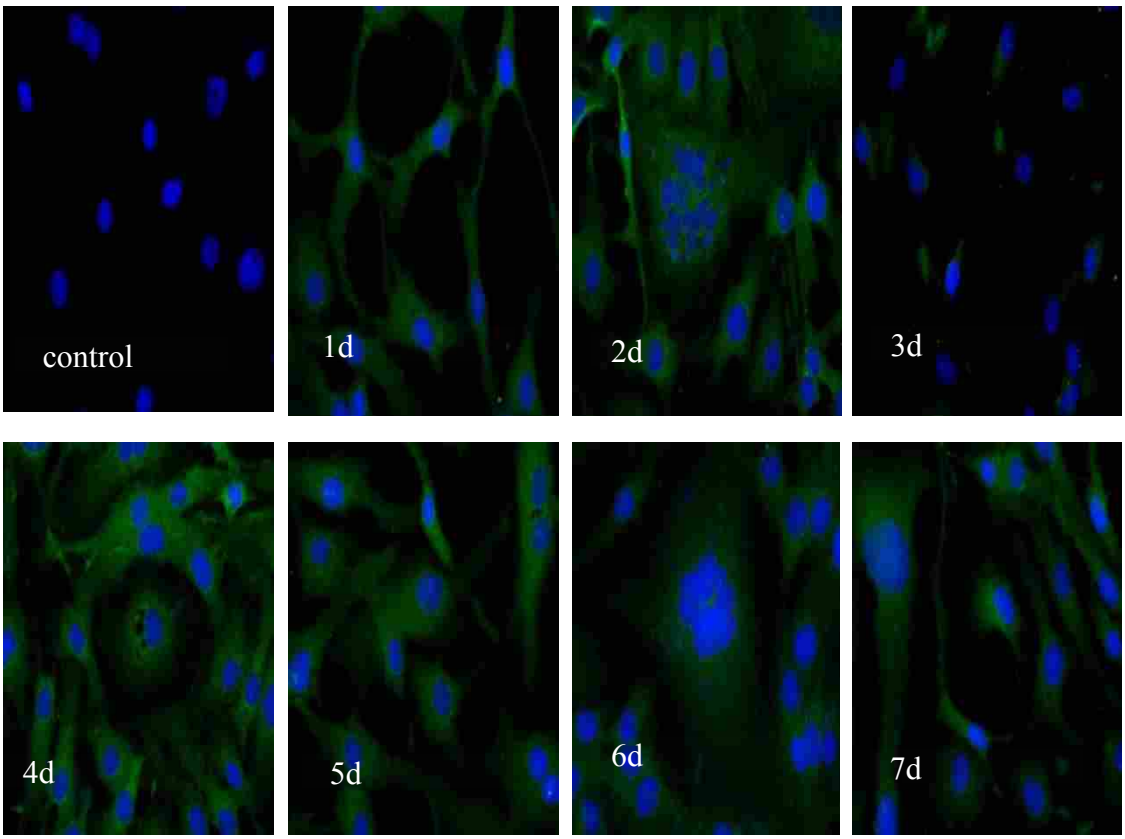


**Figure A-4: DsRNA production as determined by IF in RTgill-W1 infected with $10^{-4}$ CSV time course (1-7 days).**

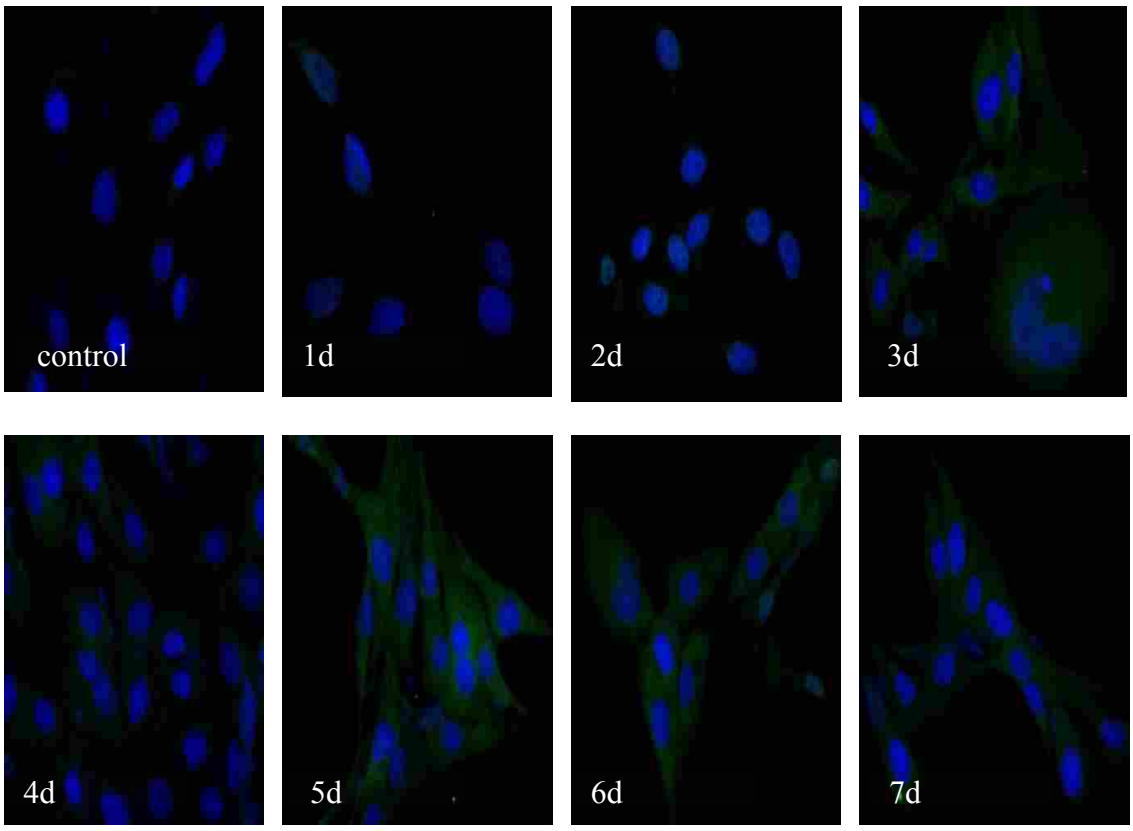


**Figure A-5: DsRNA production as determined by IF in RTG-2 infected with $10^{-4}$ CSV time course (1-7 days).**

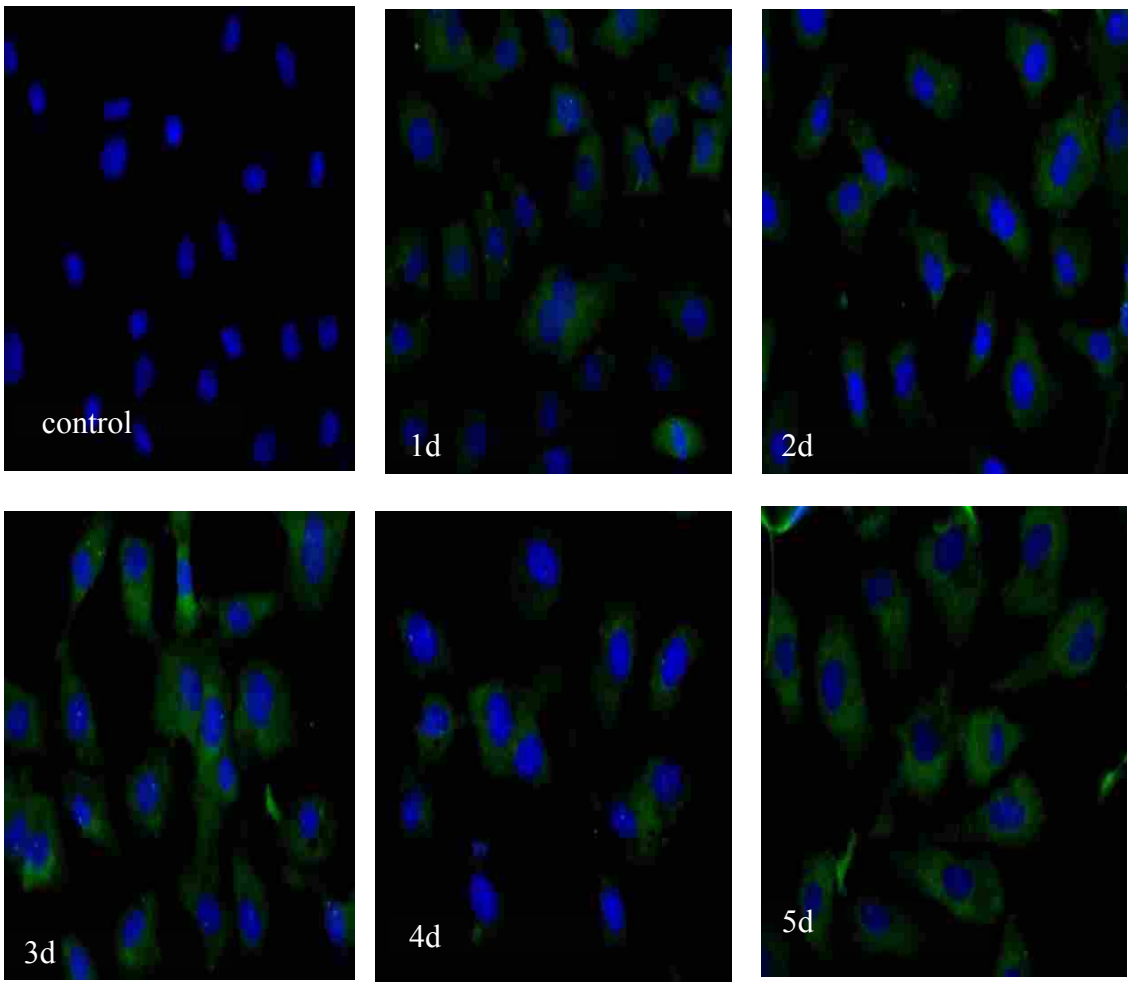


**Figure A-6: DsRNA production as determined by IF in RTgill-W1 infected with $10^{-3}$ FV3 time course (1-5 days).**

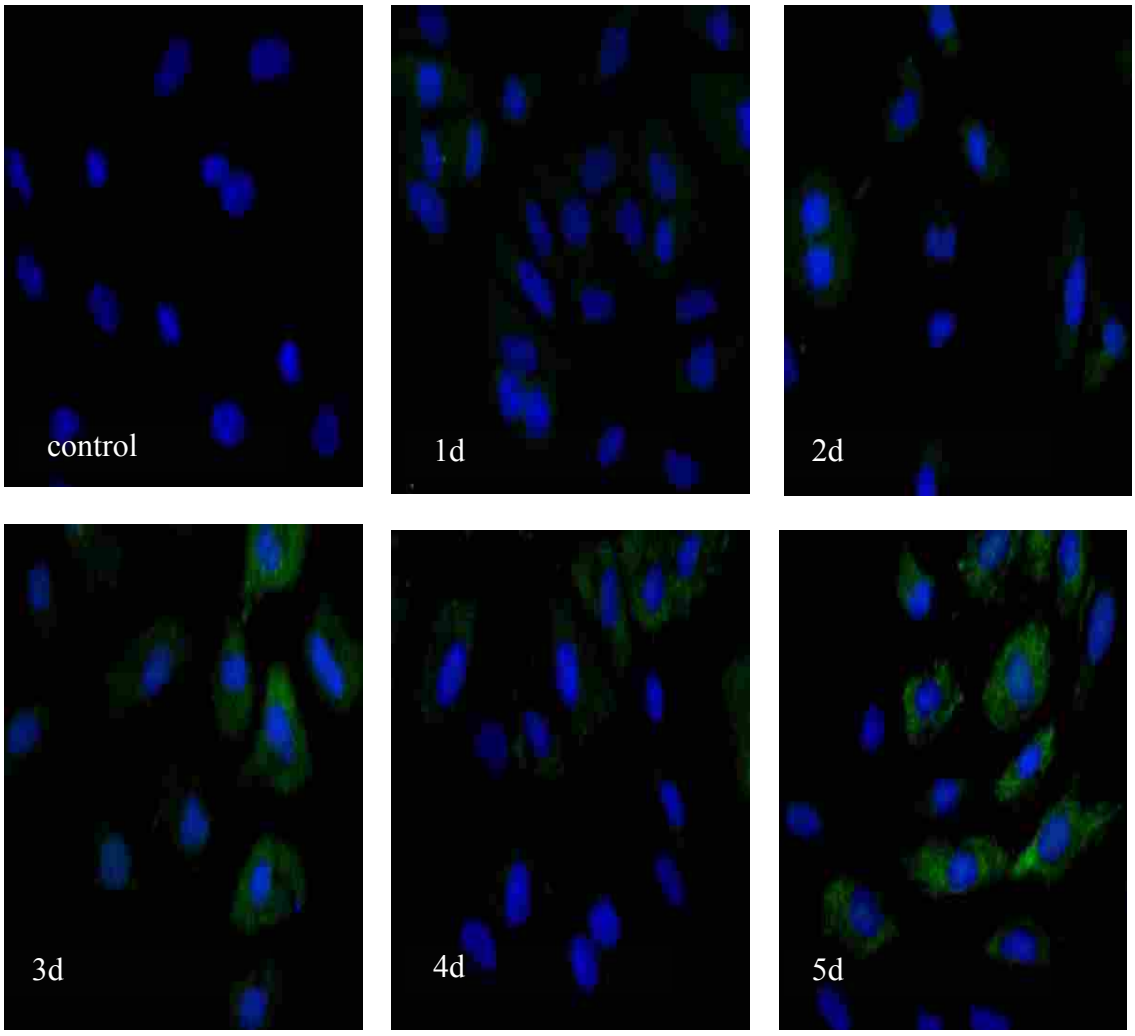


**Figure A-7: DsRNA production as determined by IF in RTgutGC infected with $10^{-3}$ FV3 time course (1-5 days).**

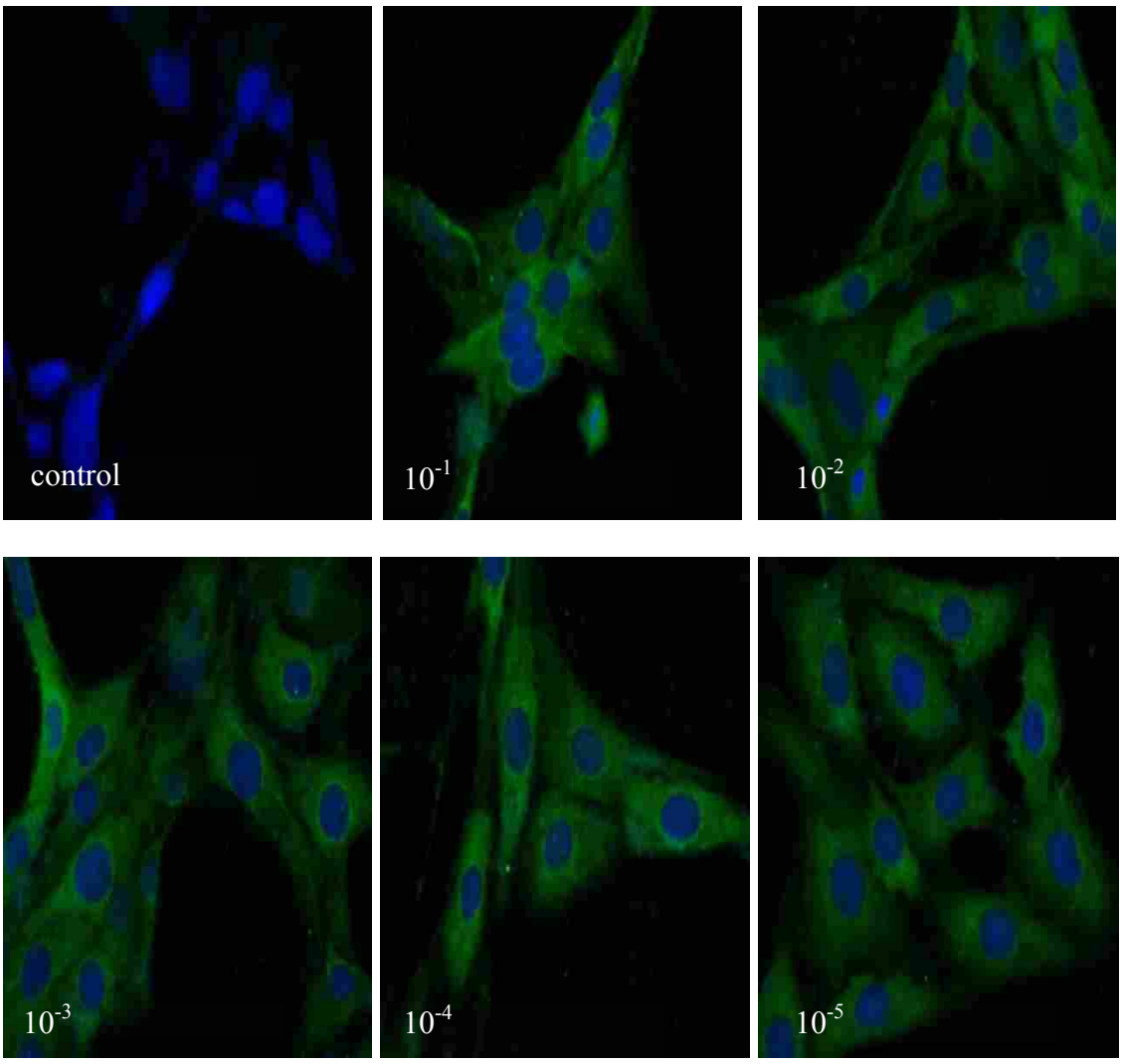


**Figure A-8: DsRNA production as determined by IF in RTG-2 infected with CSV increasing titre for 7 days.**

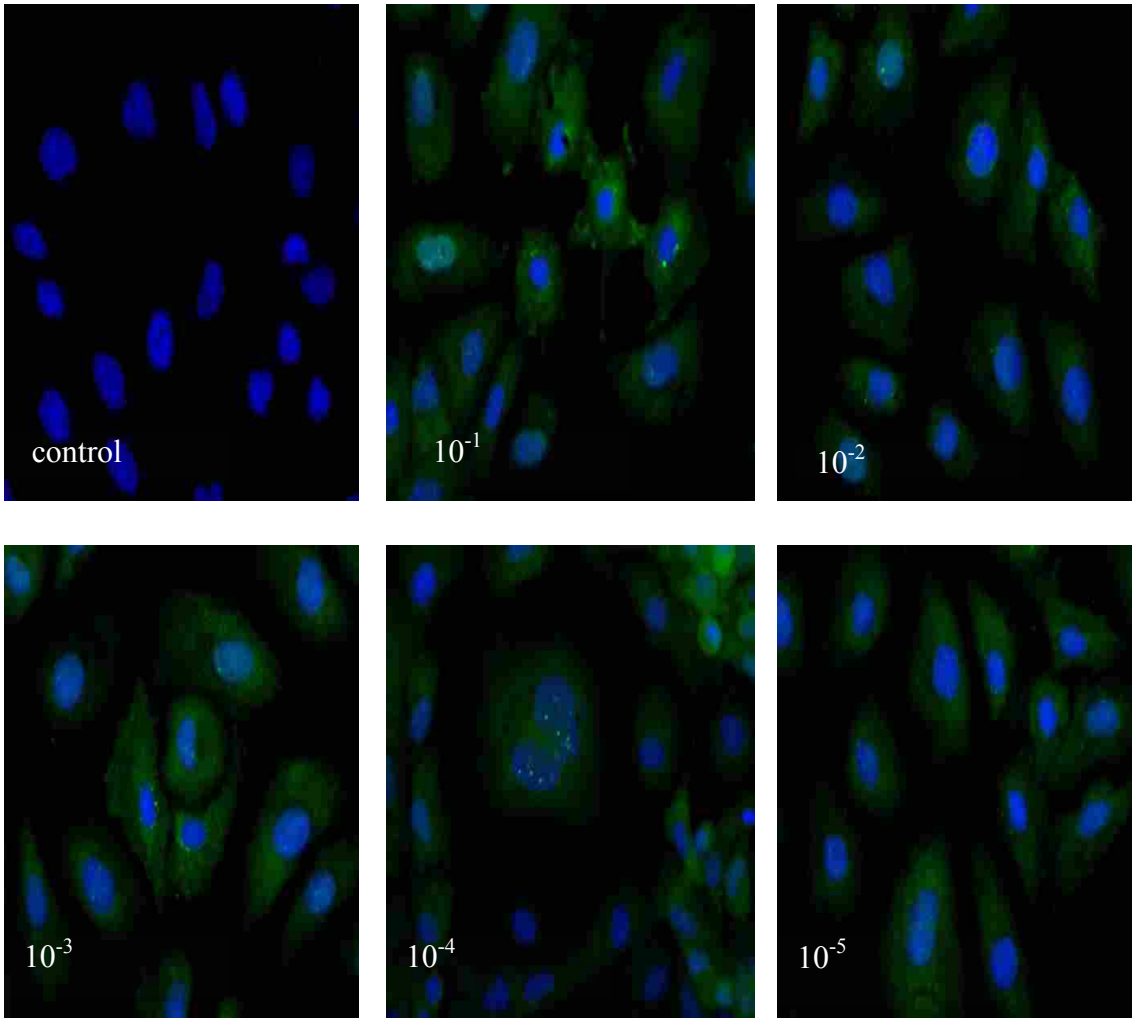


**Figure A-9: DsRNA production as determined by IF in RTgutGC infected with FV3 increasing titre for 3 days.**

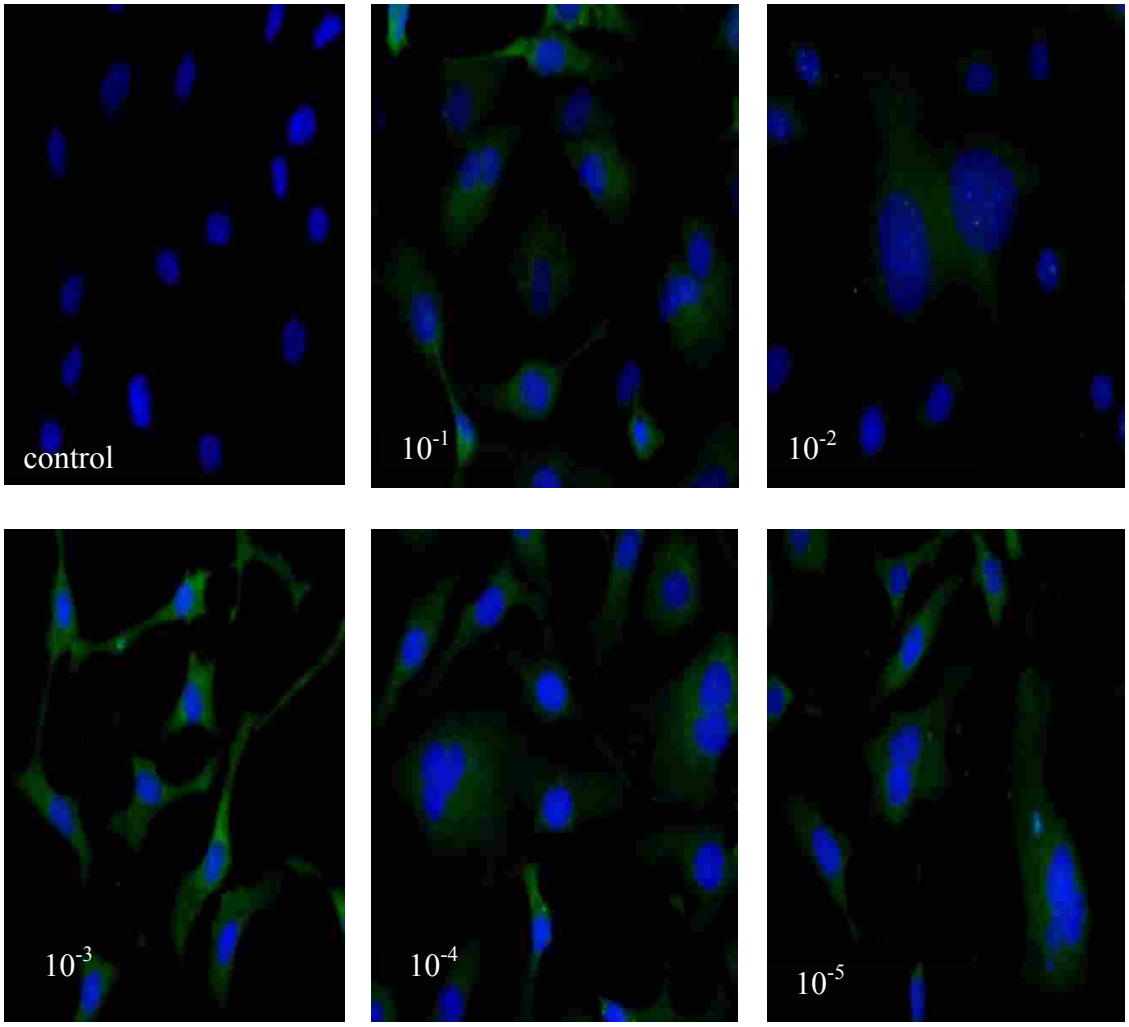


**Figure A-10: DsRNA production as determined by IF in RTgill-W1 infected with FV3 increasing titer for 3 days.**

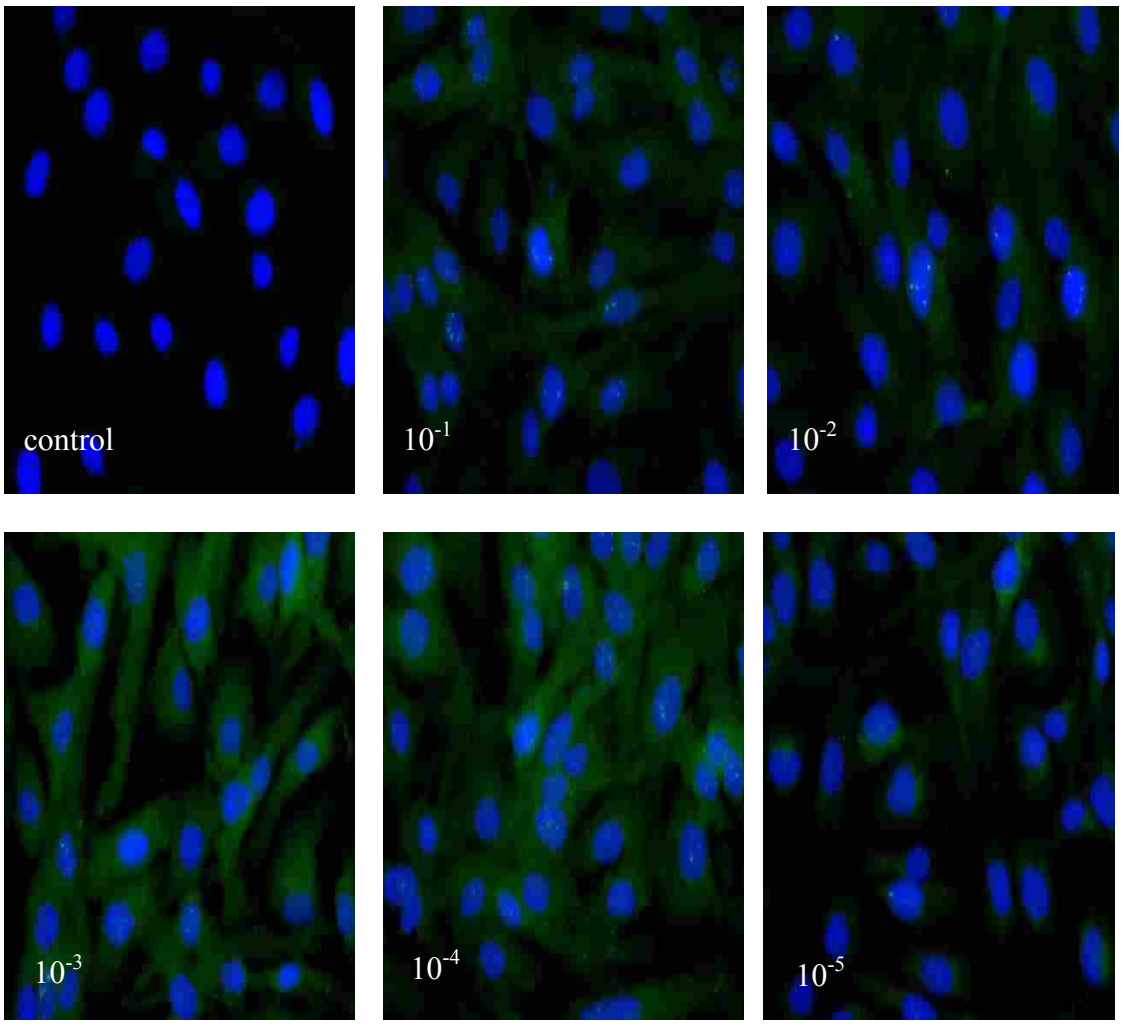


**Figure A-11: DsRNA production as determined by IF in RTgill-W1 infected with VHSV increasing titre for 5 days.**

**Appendix B. CSV infection within rainbow trout cell lines.** RTG-2 (fibroblast), RTgutGC, and RTgill-W1 (epithelial) were infected with $10^{-1}$ CSV titre for 7 days. Both the cytopathic effect and syncytia were observed after viral treatment (B), in comparison with healthy cells (uninfected) (A). All pictures were taken with the same magnification (10X).