Using cell lines to study factors affecting transmission of fish viruses

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

Factors that can influence the transmission of aquatic viruses in fish production facilities and natural environment are the immune defense of host species, the ability of viruses to infect host cells, and the environmental persistence of viruses. In this thesis, fish cell lines were used to study different aspects of these factors. Five viruses were used in this study: viral hemorrhagic septicemia virus (VHSV) from the *Rhabdoviridae* family; chum salmon reovirus (CSV) from the *Reoviridae* family; infectious pancreatic necrosis virus (IPNV) from the *Birnaviridae* family; and grouper iridovirus (GIV) and frog virus-3 (FV3) from the *Iridoviridae* family.

The first factor affecting the transmission of fish viruses examined in this thesis is the immune defense of host species. In this work, infections of marine VHSV-IVa and freshwater VHSV-IVb were studied in two rainbow trout cell lines, RTgill-W1 from the gill epithelium, and RTS11 from spleen macrophages. RTgill-W1 produced infectious progeny of both VHSV-IVa and -IVb. However, VHSV-IVa was more infectious than IVb toward RTgill-W1: IVa caused cytopathic effects (CPE) at a lower viral titre, elicited CPE earlier, and yielded higher titres. By contrast, no CPE and no increase in viral titre were observed in RTS11 cultures infected with either genotype. Yet in RTS11 all six VHSV genes were expressed and antiviral genes, Mx2 and Mx3, were up regulated by VHSV-IVb and -IVa. However, replication appeared to terminate at the translational stage as viral N protein, presumably the most abundant of the VHSV proteins, was not detected in either infected RTS11 cultures. In RTgill-W1, Mx2 and Mx3 were up regulated to similar levels by both viral genotypes, while VHSV-IVa induced higher levels of IFN1, IFN2 and LGP2A than VHSV-IVb.

The second part of the thesis examined the ability of two *Ranaviruses*, GIV and FV3, to infect non-host fish cells. This is referred to as cellular tropism and is one of many host-virus interaction events required to established successful infection in new organisms. Grouper iridovirus (GIV), belonging to the *Ranavirus* genus of the *Iridoviridae* family, was demonstrated to differentially express viral genes and induce apoptosis in three non-host fish cell lines rainbow trout monocyte/macrophage (RTS11), Chinook salmon embryon (CHSE-214) and fathead minnow *Epithelioma papulosum cyprinii* (EPC). These cells were challenged with GIV and virus entry into all three cell lines was confirmed by the expression of viral immediate early genes. The expression of the late major capsid protein gene was detected in CHSE-214 and EPC,

but not in RTS11, suggesting an earlier termination in the viral replication cycle in RTS11. Approximately 12 h after infection with GIV, cell death was prominent in all three non-host cell lines. Death was later confirmed to be apoptosis by the presence of chromosomal DNA fragmentation and phosphatidylserine externalization. To determine whether apoptosis was protein related or gene expression related, the three cell lines were infected with heat-inactivated GIV and UV-treated GIV (GIV_{UV}). The heat inactivation abolished apoptosis in all three cell lines, but each cell line responded differently to GIV_{UV}. Relative to GIV, GIV_{UV} caused no apoptosis in CHSE-214, decreased apoptosis in RTS11, and increased apoptosis in EPC. These results suggest that early GIV gene expression was needed for apoptosis in CHSE-214 but impeded apoptosis in EPC. At the cellular level, only EPC was a permissive host as EPC was the only cell line of the three capable of producing a moderate increase in virus titre. The three non-host cell lines present a good system for potentially identifying different components of GIV-induced apoptotic pathways in future studies.

Rainbow trout are not highly susceptible to frog virus 3 (FV3) induced diseases, and had been suggested to be a potential carrier for the virus. To determine which rainbow trout cell types are permissive for FV3 and act as a potential source for virus replication *in vivo*, the ability of rainbow trout cell lines from gonads (RTG-2), skin (RTHDF), liver (RTL-W1), gills (RTgill-W1), intestine (RTgut-GC) and spleen (RTS11), and primary leukocyte cultures from peripheral blood (PBL) and head kidney (HKL) to support FV3 infection was examined. RTG-2 supported a moderate level of FV3 replication while viral replication in RTL-W1 was minimal. The rest of the cell lines did not support viral replication but all succumbed to the infection and were killed by FV3. Lymphocyte-like cells from PBL and HKL were not killed by FV3 while macrophage-like cells were. Most of the cell lines died by an apoptosis-independent mechanism, presumably necrosis, while the monocyte/macrophage cell line, RTS11, died by an apoptosis-dependent mechanism. In addition, neoplastic macrophage-like human U937 cell line, and T lymphocyte-like PEER cell line were also infected with FV3 to compare their response to that of rainbow trout immune cells. U937 cells were killed by FV3 in an apoptosis-dependent manner; however, PEER T cells did not die from FV3 infection, a result similar to the lymphocyte-like fraction of rainbow trout PBL and HKL. In summary, most rainbow trout cell lines do not support significant FV3 replication; furthermore, cells of the lymphocyte origin appeared refractory to

FV3 induced cell death while those of macrophage origin underwent apoptosis as a response to FV3.

The last factor affecting the transmission of aquatic viruses examined in this thesis is the persistence of viruses in the aquatic environment. Virus persistence is influenced by natural environmental factors such as temperature, pH, desiccation and salinity, but the often unexplored anthropogenic factors can play a role. Therefore, the focus of this section was on the effect of one particular anthropogenic substance, Corexit 9500, on the infectivity of aquatic viruses with different physical characteristics. The effect of Corexit 9500, a dispersant used to clean up oil spills, on invertebrates, lower vertebrates, birds and human health have been examined but there is a significant lack of study on the effect of this dispersant on aquatic viruses. In this study, the effect of Corexit 9500 on four aquatic viruses of different structural composition was examined. Corexit 9500 reduced the titre of the enveloped viral hemorrhagic septicemia virus (VHSV) at all concentrations (10% to 0.001%) examined. The titre of frog virus 3 (FV3), a virus with both enveloped and non-enveloped virions, was only reduced at the high Corexit 9500 concentrations (10% to 0.1%). Corexit 9500 was unable to reduce the titre of non-enveloped infectious pancreatic necrosis virus (IPNV), but enhanced the titre of chum salmon reovirus (CSV) by 2-4 logs. With the ability to inactivate enveloped viruses and possibly enhance some non-enveloped viruses, Corexit 9500 has the potential to alter the aquatic virosphere.

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DEDICATION

This thesis is dedicated to my grandparents, parents and sister.

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030L	TNFR domain gene of GIV
097L	predicted E3 ubiquitin ligase domain of GIV
2% FBS/L15	L15 supplemented with 2% fetal bovine serum
7-AAD	7-aminoactinomycin D
10% FBS/L15	L15 supplemented with 10% fetal bovine serum
β-act	beta actin gene
µg	microgram
µL	microlitre
µm	micrometre
AB	alamarBlue
ANOVA	analysis of variance
ATCC	American Type Culture Collection
Bcl-2	B-cell lymphoma 2
BGMK	baby green monkey kidney
BHK	baby hamster kidney
BM	barramundi muscle
bp	base pair
CD	cluster of differentiation
cDNA	complementary DNA
CHO	Chinese hamster ovary
CHSE-214	Chinook salmon embryo
CIV	Chilo iridescent virus
CIV_{UV}	UV irradiated Chilo iridescent virus
CO_2	carbon dioxide
CPE	cytopathic effects
CSV	chum salmon reovirus
Ct	cycle threshold for real time PCR
CVPE	extract of CIV capsid protein
d	day(s)

DED	death-effector domain
DNA	deoxyribonucleic acid
DNAse I	deoxyribonuclease I
DPBS	Dulbecco's phosphate-buffered saline
EAGS	grouper spleen cells
EDTA	ethylenediaminetetraacetic acid
EF-1α	elongation factor 1α gene
EHNV	epizootic hematopoietic necrosis virus
EPC	epithelioma papillosum cyprinid
ES	embryonic stem
FBS	fetal bovine serum
fc	final concentration
FHM	fathead minnow
FL2	flow cytometer channel 2
FL4	flow cytometer channel 4
FV3	frog virus 3
g	force of gravity on earth
G	glycoprotein
GEC	grouper embryonic cells
GIV	grouper iridovirus
GIV_{uv}	UV irradiated GIV
GK	grouper kidney
h	hour(s)
HeLa	human cervical cancer cells
HKL	head kidney leukocyte
HKL-A	head kidney leukocyte adherent fraction
HKL-F	head kidney leukocyte floating fraction
HRP	horseradish peroxidase
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgT	Immunoglobulin T
IHNV	infectious hematopoietic necrosis virus
IL	interleukin
IFN	interferon
IPNV	infectious pancreatic necrosis virus
ISG	interferon-induced genes
ISVP	infectious subviral particles
Jak/STAT	Janus kinase/signal transducer and activator of transcription
kDa	kiloDalton
L	RNA-dependent RNA polymerase
L15	Leibovitz's L15 medium
L929	human fibroblastic cells
LGP2A	laboratory of genetics and physiology 2 gene
M	matrix protein
MCP	major capsid protein
MDA5	melanoma differentiation associated gene 5
MHC	major histocompatibility complex
min	minute(s)
mL	milliliter
M.O.I	multiplicity of infection
mRNA	messenger RNA
Mx	myxovirus resistance gene
n	means
N	nucleoprotein
NS	non-structural protein
NV	non-structural protein for *Novirhabdovirus*
ORFs	open reading frames
p	P-value
P	phosphoprotein

p53	protein 53
PBL	peripheral blood leukocyte
PBL-A	peripheral blood leukocyte adherent fraction
PBL-F	peripheral blood leukocyte floating fraction
PCR	polymerase chain reaction
PE	phycoerythrin
PEER	human T leukemia cell line
PI	post infection
ppb	parts per billion
PS	penicillin-streptomycin
Q3	Quadrant 3 of flow cytometer output graph
QPCR	real time PCR
RFU	relative fluorescence units
RGV	*Rana grylio* virus
RIG-I	retinoic acid-inducible gene I
RIPA	radio immunoprecipitation assay
RNA	ribonucleic acid
RNase A	ribonuclease A
RPMI-1640	Roswell Park Memorial Institute medium 1640
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	real time polymerase chain reaction
RTG-2	rainbow trout gonadal cell line
RTgill-W1	rainbow trout gill cell line
RTgut-GC	rainbow trout gut cell line
RTHDF	rainbow trout hypodermal cell line
RTL-W1	rainbow trout liver cell line
RTS11	rainbow trout monocyte/macrophage cell line
s	second(s)
SD	standard deviations
SDS	sodium dodecyl sulfate

SGIV	Singapore grouper iridovirus
SYBR Green	nucleic acid stain
TC	tissue culture
$TCID_{50}$	tissue culture infectious dose
TE	Tris-EDTA
T_H2	T helper 2
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
Tris	tris (hydroxymethyl) aminomethane
U937	human neoplastic monocytic cell line
UV	ultraviolet
v/v	ratio of volume to volume
VHSV	viral hemorrhagic septicemia virus
VHSV-I	VHSV genotype-I
VHSV-IV	VHSV genotype-IV
VP	Birnavirus genes
VSV	vesicular stomatitis virus
w	water control
XTC-2	frog epithelial cell line

CHAPTER 1

General introduction

1.1 Introduction

The transmission of aquatic viruses in the natural environment and in aquaculture facilities is influenced by many factors including host defenses, cellular tropism, and environmental survival. In this thesis different aspects of these factors are studied using fish cell lines and several fish viruses. The viruses that are used in this study are viral hemorrhagic septicemia virus (VHSV) from the *Rhabdoviridae* family, chum salmon reovirus (CSV) from the *Reoviridae* family, infectious pancreatic necrosis virus (IPNV) from the *Birnaviridae* family, and grouper iridovirus (GIV) and frog virus 3 (FV3) from the *Iridoviridae* family. To evaluate the role of host defenses in cellular tropism and viral transmission, host immune and non-immune cells are infected with viruses of varying virulence and evaluated for expression of antiviral genes. To study the role of cellular tropism on virus transmission, viruses of one host are used to infect different cell types from a different host to determine if the potential for cross species transmission, or host shift, exists at the cellular level. In studying the environmental survival of viruses, one specific anthropogenic problem, the effect of oil spill dispersant, Corexit 9500, on the survival and infectivity of viruses was examined. A graphical overview of the purpose outlined here can be seen in Fig. 1.1 along with the virus chosen to be studied within each factor that influences viral transmission.

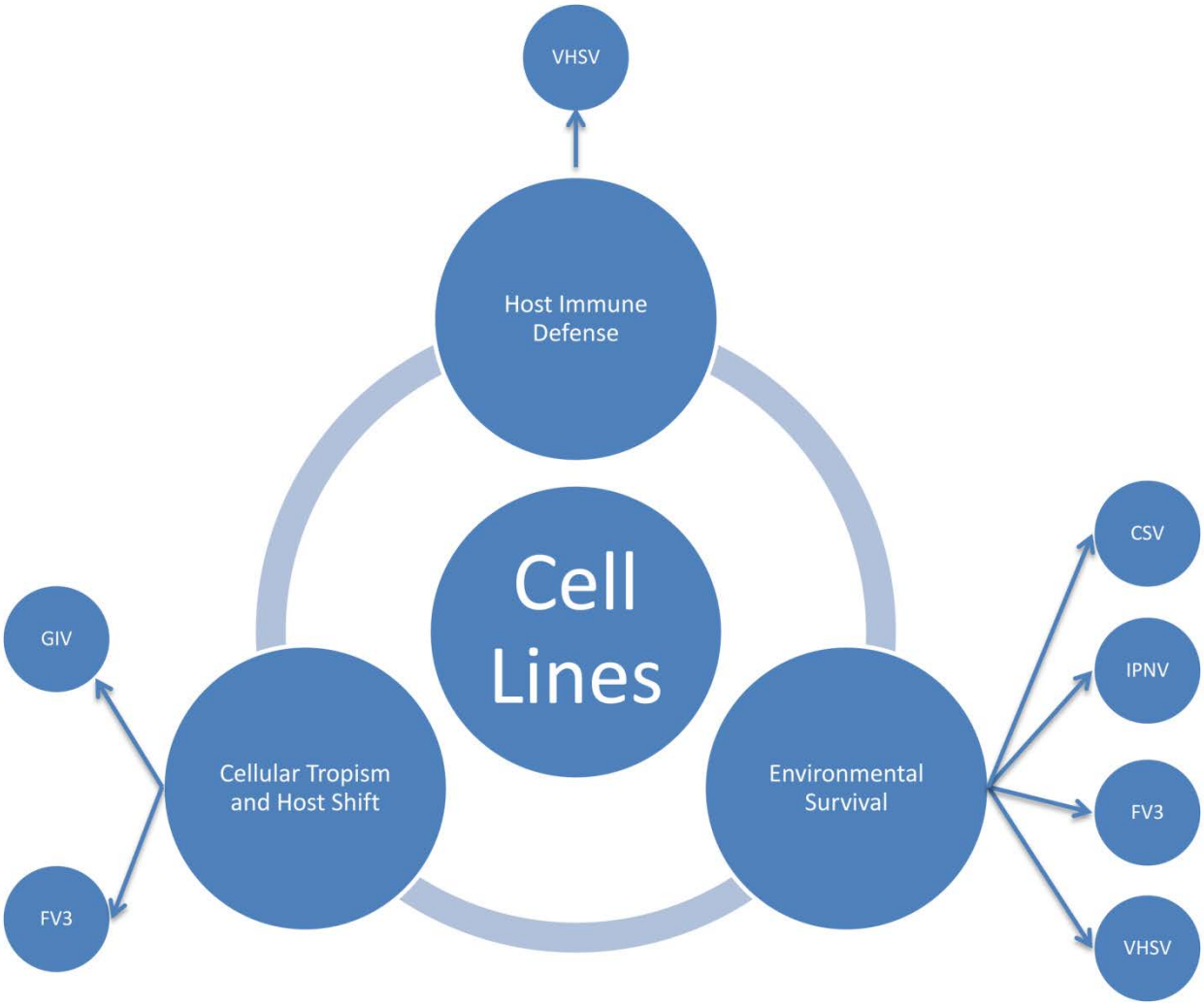

Fig. 1.1 Overview of purpose

A graphical overview of the use of cell lines to study factors affecting the transmission of aquatic viruses. The virus of focus is indicated for each factor being studied.

1.2 Fish cell lines and the study of fish viruses

The development of fish cell lines has greatly advanced the field of fish virology in a number of ways. In basic research, it allowed researchers to study the interaction between viruses and cells based on specific cell types. Cell lines provide a more homogenous population of cells leading to more consistent and reproducible results than primary cultures [1]. They also allow for viruses to be conveniently propagated and amplified for either storage or further downstream studies of viral properties and structures. For application based work, cell lines are used as diagnostic tools to isolate and diagnose disease causing viruses in the environment and in aquaculture [2]. One of the earliest fish cell lines to be developed was RTG-2, a rainbow trout gonadal cell line, which was used to study IPNV since the cell line is highly susceptible to the virus [3]. Four other common fish cell lines used to diagnose fish diseases are bluegill fry (BF-2), Chinook salmon embryo (CHSE-214), fathead minnow (FHM) and *epithelioma papillosum cyprinid* (EPC); CHSE-214 is recommended for diagnosis of IPNV and salmonid alphavirus while the rest are for VHSV and infectious hematopoietic necrosis virus (IHNV) [4]. EPC have recently been reclassified as originating from fathead minnow instead of carp [5]. Many other cell lines have been developed to study other economically important fish viruses such as infectious salmon anemia virus, nervous necrosis virus and epizootic hematopoietic necrosis virus [2].

In addition to these commonly used cells lines from diverse fish species, many rainbow trout cell lines have been previously derived from various tissue locations and are used for the study of viruses in this thesis; four adherent cell lines were previously derived from tissue of non-immune origins and one semi-adherent cell line of immune origin. The four adherent rainbow trout cell lines used were the epithelial-like RTgill-W1 from the gill [71], RTL-W1 from the liver [72], RTgut-GC from the intestine [73] and RTHDF (hypodermal connective tissue) from skin [74]. The non-adherent cell line used was the monocyte/macrophage-like, RTS11, from the spleen, which grows loosely over the culture surface [75].

1.3 Viruses

1.3.1 *Rhabdoviridae* and Viral Hemorrhagic Septicemia Virus

The *Rhabdoviridae* family is phylogenetically divided into nine different genera: *vesiculovirus*, *lyssavirus*, *ephemerovirus*, *novirhabdovirus*, *cytorhabdovirus*, *perhabdovirus*, *sigmavirus*, *tibrovirus* and *nucleorhabdovirus* [6]. The host range of the *Rhabdoviridae* family is large, spanning plants, insects, fish, birds, and mammals. The genomes of these viruses are composed of a negative single-stranded linear RNA, and all encode five structural genes, nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L) in order from 3' to 5', in addition to some non-structural genes of mostly uncertain function in a few viruses of different genera [7]. Some *Rhabdoviruses* contain non-structural genes located between the G and L genes, for example, the NV gene in *novirhabdovirus* [8] and open reading frames (ORFs) of genes in *ephemerovirus* [7]. Other rhabdoviruses contain non-structural genes between the P and M regions in addition to other ORFs that overlap with the N and G genes [7]. Structurally, rhabdovirus virions are enveloped with bullet morphology, although plant Rhabdoviruses can be bacilliform; the size of the virions range from 100-430 nm in length and 45-100 nm in diameter [7]. The replication cycle of most rhabdoviruses begins with the binding of viral G proteins to cellular receptors and initiation of receptor-mediated endocytosis; however, plant rhabdoviruses enter cells directly as a result of mechanical damage caused by insect vectors [7]. Upon endocytosis, acidification of the endosome leads to fusion of the viral membrane with the endosomal membrane and release of the viral genome into the cytoplasm where viral transcription, translation, and genome replication occur. Virions are assembled at and budded out from the cellular membrane [7].

Viral hemorrhagic septicemia virus is a fish rhabdovirus that belongs to the *Novirhabdovirus* genus. It causes viral hemorrhagic septicemia disease in salmonid fish with great economic impact. The virus can be classified into four different genotypes: I, II, III and IV, based on phylogenetic analysis of the N and G genes [9]. Of interest to this project are VHSV in genotype I and IV. Genotype I can be further sub-categorized into groups "a", "b", "c", "d", and "e", where viruses in group Ia are highly virulent to rainbow trout and viruses in the other subgroups are generally less virulent [9]. Genotype IV can be sub-divided into groups "a" and "b" [10]. VHSV genotype IVa is highly virulent to Pacific herring and was first identified in marine fishes

in the 1990s [11]. VHSV genotype IVb is found in the North American Great Lakes and caused multiple fish kills in different fish species [10,12,13]. Both VHSV genotype IVb and IVa are generally not very pathogenic toward rainbow trout [14,15].

1.3.2 *Reoviridae* and Chum Salmon Reovirus

The *Reoviridae* family is a big family of viruses that is divided into two subfamilies and a total of 15 genera. The two subfamilies are the *Sedoreovirinae* and the *Spinareovirinae*. The *Sedoreovirinae* subfamily consists of six genera: *Cardoreovirus*, *Mimoreovirus*, *Orbivirus*, *Phytoreovirus*, *Rotavirus*, and *Seadornavirus*. The *Spinareovirinae* consists of nine genera: *Aquareovirus*, *Coltivirus*, *Cypovirus*, *Dinovernavirus*, *Fijivirus*, *Idnoreovirus*, *Mycoreovirus*, *Orthoreovirus*, and *Oryzavirus* [6]. Viruses in the *Reoviridae* family can infect plants, insects, crustaceans, fish, birds, and mammals [16]. In general, members of the *Reoviridae* family contain genomes ranging from 10-12 segments and 6-12 structural virion proteins [17]. The genome segments are classified into three classes based on size and migration on polyacrylamide gel electrophoresis, and they are large, medium, and small. For example, the ten-segment genome of the *Orthoreovirus* genus is divided into three large, three medium and four small segments, and the viral proteins can also be classified based on size as λ for large, μ for medium and σ for small [17]. Structurally, reovirus virions are icosahedral in shape, ranging from 60-80 nm in size, and the virus genome-protein core is encapsulated by two protein capsids, an inner and outer layer [17]. Reovirus enters host cells by receptor mediated endocytosis through the binding of protein spikes on the outer protein capsid to sialic acid receptors on the cell membrane; within the cell, the endosomes fuse with lysosomes. The resulting acidification of the endosome and proteases from the lysosome degrade the outer capsid, creating subviral particles. These particles are delivered to the cytoplasm where the virus replication and assembly occur [17].

Chum salmon reovirus (CSV) is a fish reovirus that belongs to the *Aquareovirus* genus. Similar to other reoviruses, the CSV genome is composed of 11 segments of double-stranded RNA that is surrounded by two protein capsids [18]. For the CSV genome, three segments are classified as large, three as medium and five as small. CSV virions contained five major structural proteins: two large, one medium and two small proteins, and some minor capsid proteins [18]. A more detailed analysis of another *Aquareovirus*, striped bass aquareovirus,

revealed that it contained seven structural proteins (VP1-VP7) and five non-structural proteins (NS97, NS39, NS29, NS28, and NS15); each of the genome segments encoded one protein except for segment 11 which encoded two proteins [19]. With regards to infectivity, CSV can replicate and produce infectious titre in Chinook, chum and kokanee salmon fry but is not very pathogenic and causes no significant mortality to those fish. Many cell lines can support CSV replication, including the Chinook salmon embryonic cell line (CHSE-214) and the reclassified fathead minnow cell line *epithelioma papulosum cyprini* (EPC) [18,20].

1.3.3 *Birnaviridae* and Infectious Pancreatic Necrosis Virus

The *Birnaviridae* family consists of four genera: *Aquabirnavirus*, *Avibirnavirus*, *Blosnavirus*, and *Entomobirnavirus* [6]. Viruses in this family can infect insects, birds and many aquatic species [21]. The genomes of Birnaviruses are composed of two double-stranded RNA segments (A and B). The virions are non-enveloped with a single icosahedral protein capsid, approximately 60-70 nm in diameter and are composed mainly of four structural proteins, large size VP1 (94 kDa), medium size VP2 (54 kDa), small size VP3 (31 kDa) and VP3a (also known as VP4) (29 kDa) [22,23]. VP1 functions as the viral RNA-dependent RNA polymerase, VP2 and VP3 as capsid proteins and VP4 as a protease involved in cleaving the precursor of VP2 [23]. The virus enters host cells by receptor-mediated endocytosis using capsid protein VP2 to bind cellular receptors [22].

Infectious pancreatic necrosis virus (IPNV) is the type species of *Birnaviridae* family and belongs to the *Aquabirnavirus* genus. This virus can be pathogenic to economically important salmonid fish such as rainbow trout, brook trout and Atlantic salmon [24].

1.3.4 *Iridoviridae* and Grouper Iridovirus and Frog Virus 3

The family *Iridoviridae* is divided into five genera: *Chloriridovirus*, *Iridovirus*, *Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus* [6]. Viruses in the *Iridoviridae* family infect insects, crustaceans, teleost fish, amphibians and reptiles [25]. These viruses contain a large double-stranded DNA genome with size ranging from 105-212 kbp, and the genome encodes 92-211 putative proteins [25]. Their genomes are circularly permuted and terminally redundant, which is one reason why they have large genome sizes as the redundant portion of the genome can account for up to 50% of total genome length [26]. Structurally, virions of this virus family

can be both enveloped and non-enveloped and icosahedral in shape [26]. The enveloped virions can enter host cells by endocytosis pathways, either receptor-mediated or caveola-mediated; non-enveloped virions enter host cells by fusing with the cell membrane [25]. Regardless of the route of entry, the genome is initially transported to the cell nucleus where the virus can begin a two-stage replication cycle. In the first stage, inside the nucleus, virus immediate-early and delayed-early genes are transcribed and the genome is replicated. In the second stage, the newly synthesized genomes are transported out into the cytoplasm where they form concatamers and late viral genes are transcribed. Viral protein synthesis occurs in the cytoplasm where virion assembly also occurs. The assembled virions accumulate in the cells in paracrystalline array networks. Viral egress occurs either by the virions budding from the cell membrane, forming enveloped virions, or by cell lysis, releasing non-enveloped virions [25].

Grouper iridovirus (GIV) and frog virus 3 (FV3) belong to the *Ranavirus* genus where FV3 is the type species [25]. GIV infects many species of grouper that are economically important to East Asian countries [27] and FV3 infects amphibians [28]. GIV was originally isolated from a grouper farm in southern Taiwan [29] and FV3 from leopard frogs [30].

1.4 Viral host defenses in fish

The fish defense system against pathogens consists of both adaptive and innate immunity, which is similar to that in mammals. The adaptive immune system of fish contains many components that parallel those of mammals such as immunoglobulins, lymphocytes (T and B cells) and major histocompatibility complex class I and II [31]. However, some differences exist. Mammals have immune organs such as spleen, thymus and bone marrow whereas fish have thymus, head kidney and spleen [31,32]. Fish lack secondary lymphoid organs such as germinal centers and lymph nodes that are present in mammals. With regards to immunoglobulin, fish have three classes (IgM, IgD and IgT) whereas mammals have five (IgA, IgD, IgE, IgG and IgM); in addition, mammals are capable of antibody class switching while fish are not [31]. Although fish have an adaptive immune system, the innate immune system is believed to play a more important role in protecting fish, especially because fish are constantly exposed to the external environment during early developmental stages where adaptive immunity may not be fully developed [32].

The fish innate immune system and its many components were evolved to fulfill the following general roles in protecting fish from pathogen infection. These roles are prevention, detection, inform and response. The first key step in protecting an organism from infection is prevention, which can be accomplished by the use of physical barriers such as mucus to protect locations on fish like the skin and gills that can serve as potential portals of entry for pathogens. The mucus functions by trapping pathogens and degrading them with lysozymes and proteases [33]. Fish mucus also contains antimicrobial peptides; however, few studies have examined fish antimicrobial peptides and the ones that did showed that the synthetic dsRNA viral minic (poly I:C) was capable of upregulating the expression of Cathelicidin, β-defensin, and Hepticidin in rainbow trout cells [33].

When pathogens penetrate the physical defenses and enter cells, the next role of the innate immune system inside cells is to detect the foreign intruders. In the evolutionary arms race between pathogens and cells, the cells have evolved many different sensors to detect common structural components that are shared between similar classes of pathogens, called pathogen-associated molecular patters. For bacterial pathogens, these components are lipopolysaccharide, peptidoglycan, flagellin and unique bacterial DNA and RNA structures, and for viral pathogens, they are commonly non-capped single or double-stranded RNA [70]. To detect these pathogen signatures, the cells employ many different families of sensors (receptors). Commonly known cellular pathogen sensor families are the Toll-like receptors (TLR), the retinoic acid inducible gene 1-like receptors (RLRs), and the nucleotide-binding domain, leucine-rich repeat containing proteins receptors (NLRs) [70]. The TLR family contains many different members tasked with recognizing various pathogen signatures; TLR1 and TLR2 recognize bacterial lipopeptides and peptidoglycan, respectively; TRL3 short dsRNA; TLR4 lipopolysaccharide; TLR5 flagellin; TLR7 and TLR8 ssRNA; TLR9 CpG DNA; and in fish, TLR22 recognizes long dsRNA [70]. The RLRs family consists of cytosolic receptors retinoic acid inducible gene protein I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5); RIG-I recognizes short dsRNA while MDA5 long dsRNA [70]. Finally, the NLRs family consists of NOD-like receptors that recognize diverse pathogen signatures, including lipopolysaccharide, peptidoglycan and nucleic acids [70].

After recognition of pathogens, the cell sensors begin signaling cascades to alert and inform nearby cells and the rest of the innate immue system of the danger. Signals from the cellular

sensors lead to the induction of the interferon system. The interferon system consists of a set of interferon genes that participate in a signal transduction pathway that leads to the activation of antiviral genes, or interferon-induced genes (ISGs). There are three types of interferon, type I, II and III; and type I and II can be induced by viruses [45]. The expressed type I interferon activates its own signaling cascade, the Jak/STAT pathway, that results in the expression of many ISGs [33]. Well known ISGs are the set of Mx genes that were originally discovered in mice and shown to have antiviral activity against influenza and vesicular stomatitis virus. Mx homologues are also present in various fish species and have been demonstrated to have antiviral activity against different fish viruses, including IPNV [45].

Once the rest of the innate immune system has been alerted, the next step is for the innate immune system to respond by eliminating the pathogens and infected cells, and signaling the adaptive immune system. Elimination of extracellular bacterial pathogens can be done by recruitment of neutrophils and macrophages, which can phagocytize and destroy the pathogens. In some virus infections, the infected cells can be eliminated intrinsically by the cytosolic induction of cellular apoptosis through Protein Kinase R recognition of viral dsRNA or extrinsically by the recruitment and action of Natural Killer cells [33]. Natural Killer cells are cells of the innate immune system that can recognize virus infected cells (by a currently unclear mechanism) and kill them by osmotic lysis and apoptosis with perforin and granzyme [33]. Finally, the adaptive immune system is activated by antigen presenting dendritic cells and macrophages [33]. Since macrophages have many diverse roles in development and immune response and are the main cells of focus in this thesis, the role of macrophages in virus-vertebrate host interactions is further discussed.

1.4.1 Role of macrophage in virus-vertebrate host interactions

Macrophages have multiple key roles in the interaction of viruses with vertebrate hosts, and the interaction between the macrophage and viruses can either favor the host or the viruses. The kidney is where the majority of haematopoiesis occur in teleost fish and the spleen is the site where macrophages mature [34]. Aside from these locations, macrophages are also found in the intestinal tract, the liver and the gills [35,36]. The intestinal tract and gills are considered as external environment and presence of macrophages in these areas suggests that they are there as early responders to infection. Macrophages have very diverse functions and fish macrophages

have been suggested to have four activation states: innate activation by microbial stimulus and toll-like receptors, classical activation by IFNγ and TNFα, alternative activation by T_H2 cytokines IL-4 and IL-13 that can be identified by arginase activity, and regulatory activation that requires IL-10 [37]. During an innate immune response, virus infections of macrophages can lead to the induction of antiviral genes such as TNFα and IL-1β [38,39]. Macrophage can also endocytose viruses and destroy them in a respiratory burst response by generating reactive oxygen species and reactive nitrogen species such as nitric oxide [38,40]. During the adaptive immune response, macrophages also function as antigen presenting cells; through the MHC-II pathway macrophages can present viral antigens to B-lymphocytes and $CD4^+$ T cells, and through the MHC-I pathway, macrophages can cross-present antigens to $CD8^+$ T cells [41,42]. Some macrophage-virus interactions can favour the survival and distribution of viruses at the expense of the host organism. For example, monocytes and macrophages can act as "Trojan horses" to traffic viruses such as human immunodeficiency virus, simian immunodeficiency virus or lactate dehydrogenase-elevating virus from one location to another in the host [43,44].

1.4.2 The role of cell death in virus-vertebrate host interactions

The death of cells can be morphologically defined by one of the following criteria, loss of plasma membrane integrity, fragmentation of the nucleus and cells to produce smaller bodies (apoptotic bodies), or engulfment by neighboring cells [46]. Although the end results are 'dead' cells, there can be many ways for cells to die. Two traditionally recognized and well defined forms of cell death are necrosis and apoptosis. There are many other modes of cell death that have also been characterized; they are cornification, mitotic catastrophe, anoikis, excitotoxicity, Wallerian degeneration, paraptosis, pyroptosis, pyronecrosis, and entosis [46].

Apoptosis is a form of orderly cell death where the steps leading to the death of cells follow well defined patterns. There are three cellular pathways that can trigger a signaling cascade, leading to apoptosis: 1) the extrinsic pathway, where extracellular ligands bind to cellular membrane receptors (the death receptors); 2) the intrinsic pathway, where exposure of cells to stressors such as viruses, toxins, and other damaging agents leads to a loss in mitochondrial membrane potential; and 3) the perforin/granzyme pathway triggered by cytotoxic T cells and natural killer cells on compromised target cells [47]. Morphologically, apoptosis can be characterized by cell shrinkage, chromatin condensation, and formation of subcellular bodies;

biochemically, apoptosis is defined by DNA fragmentation, externalization of phosphatidylserine on the cell membrane and activation of the initiator and executioner caspase cascade [47].

The battle for control over apoptosis during virus infections of host cells can determine the outcome of infections in host organisms. Many viruses can either suppress or induce apoptosis in host cells or do both but at different times during infections. During early stages of infections, suppression of apoptosis is beneficial for viruses to allow enough time for viral gene expression, genome replication and virion assembly. There are many viral strategies to suppress apoptosis. Viruses encode mimics of cellular death receptors or death signaling domains, such as the DED domain; these mimics competitively inhibit the cellular counterparts [48]. Viruses can inhibit caspases or the tumour suppressor protein, p53, or express anti-apoptotic Bcl-2 homologs [48]. In contrast to suppressing apoptosis, in other situations, the induction of apoptosis is beneficial for viruses. Once viruses have completed their replication cycles inside the cells, apoptosis can be induced to promote the release and dissemination of viral progenies; the virions disseminated in apoptotic bodies are quickly taken up by neighbouring cells and at the same time, reducing host inflammatory responses [48]. Viruses can further suppress the immune system by inducing apoptosis in immune cells such as macrophages [49]. One example of a virus family that can both suppress and induce apoptosis is the *Iridoviridae* family; viruses in this family express an anti-apoptotic Bcl-2 homologue [50] but at the same time, have been frequently reported to induce apoptosis in infected cells [25].

1.5 Viral tropism, entry and host shifts

Viral tropism is defined as the ability of viruses to productively replicate in host cells, tissues or organisms. As such, for viruses to establish successful infections in hosts they have to accomplish the following tasks: cell entry, the ability of viruses to enter cells through various mechanisms; cell compatibility, the ability of viruses to use cellular machinery for replication; and immune evasion or suppression, the ability of viruses to evade or overcome host defenses at the cellular, tissue, and whole organism level [51]. The completion of these requirements during encounters with new species results in host shifting events. Viruses can enter cells by either directly fusing with the cell membrane or by binding to membrane receptors and initiating endocytosis [52]. Viruses that enter cells through endocytosis can use a variety of endocytic

pathways: clatherin-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis, and phagocytosis [53].

Entry of viruses into cells is the initial necessary step for any successful viral infection but it alone does not guarantee successful replication. Viruses must be able to initiate viral genomic transcription and genome replication inside the host and use host translational machinery for protein synthesis. For example, while IPNV is capable of entering mammalian rabbit kidney cells at the cellular level, it could not replicate in them [54]. In contrast, *Aquareoviruses* and fish rhabdoviruses, such as VHSV and IHNV, were shown to cause cytopathic effects in mammalian cell lines and successfully replicate [55,56]. Successful replication of viruses in a particular host cell type does not guarantee that viruses can establish a successful infection in host organisms as host antiviral defenses such as antiviral cytokines and immune cells can play a role in suppressing infection. The cell types that viruses come into contact with matters as viruses may be capable of infecting one type of cell in hosts but not another type of cell in the same host. For example, a VHSV genotype Ia isolate was able to complete its replication cycle in a rainbow trout fibroblast cell line, RTG-2, but its infection was aborted in the monocyte/macrophage cell line, RTS11 [57].

Ranavirus, *Novirhabdovirus*, and *Aquabirnavirus* have either recent host shifting events or have a very broad host range. *Ranavirus* has been recently demonstrated to have host shifted between fish, frogs, reptiles and salamanders; however, the ancestral *Ranavirus* is suggested to have originated from fish [58]. Both VHSV in the *Novirhabdovirus* genus and IPNV in the *Aquabirnavirus* genus have very broad host range and have been isolated from at least 70 and 80 different fish species, respectively [59].

1.6 Natural and anthropogenic inactivation of aquatic viruses in the environment

Environmental survival or inactivation of aquatic viruses may have roles in virus transmissions. The persistence of viruses in the environment increases the possibility of coming into contact with potential hosts. Previous studies of aquatic virus survival have looked at traditional factors like temperature, pH, desiccation, and salinity. For example, exposure of CSV to low pH (approximately pH 3) increased infectivity, whereas a temperature of 56 ^{o}C abolished infectivity [20]. Relative to VHSV, IPNV survived longer at 60 ^{o}C whereas VHSV was greatly inactivated after one hour [60]. At a high pH of 12, IPNV was completely inactivated within 60

min while VHSV was not but did show inactivation by six hours; however, both IPNV and VHSV survived better at the low pH of four than at high pH [60]. Studies on both the effects of temperature and desiccation on VHSV showed that the virus remained infectious under drying conditions at room temperature and below for at least ten days and at 26 ^{o}C for at least six days but became almost completely inactivated after only one hour at 37 ^{o}C [61]. Further studies showed that under wet conditions, VHSV attached to anthropogenic materials such as fishing lines, aluminum cans, and plastic water bottle pieces for at least ten days and these served as potential fomites for the transmission of the virus, but under drying condition the transmission potential is greatly reduced to less than six days [62]. FV3 was shown to be resistant to desiccation and it also survived for 22 to 31 days at 20 ^{o}C and 58 to 72 days at 4 ^{o}C in unsterile pond water habitats; in soil, FV3 survived from 30 to 48 days [63]. Hawley and Garver [64] studied the effects of salinity on the survival of VHSV isolates by comparing virus survival rate in freshwater to seawater. They found that in raw freshwater VHSV survival ranged from 40 days at 4 ^{o}C to less than one day at 30 ^{o}C [64]. In raw seawater, VHSV survival was lower, 13 days at 4 ^{o}C and less than two days at 20 ^{o}C [64]. In addition, there was no correlation between the origin of the VHSV isolates and their survival rates in freshwater or seawater [64].

There have been approximately 20 major oil spills into the aquatic environment from oil tankers since 1970s [65]. In 2010, the Deepwater Horizon oil rig explosion released approximately 757 million litres of crude oil into nearby aquatic environments [66]. Current methods to clean up aquatic oil spills are 1) burning oil, 2) physical removal of oil by boats, and 3) application of chemicals like dispersant and sorbants [67]. The main method used to clean up the Deepwater Horizon oil spill was application of dispersants, where approximately 7.57 million litres of oil dispersants, mainly Corexit 9500, were used to disperse oil [66]. Oil dispersants function by breaking down large pools of oil into small oil droplets, allowing the particles to sink into the water column; the small oil particles are then diluted by waves into very minute concentrations [68]. The effects of crude oil on viruses have been previous examined for VHSV, which arose from the concern of the Exxon Valdez oil spill on the survival of aquatic viruses; VHSV was found to be unaffected by crude oil at concentrations of 3 to 5 ppb [69]. However, to date, there have been no published studies on the effects of any oil dispersants on aquatic viruses.

1.7 Objectives of the thesis

The objectives of the thesis are illustrated in the following questions:

A. Do two strains of VHSV preferentially infect epithelial cells from the gill or macrophages from the spleen of rainbow trout? (Chapter 2)

B. Does GIV infect cells from several non-host species? (Chapter 3)

C. Does FV3 infect different cell types from a single non-host species, rainbow trout? (Chapter 4)

D. Do four different viruses infecting fish respond similarly or differently to a commonly used oil spill dispersant? (Chapter 5)

CHAPTER 2

Differential effects of viral hemorrhagic septicemia virus (VHSV) genotypes IVa and IVb on gill epithelial and spleen macrophage cell lines from rainbow trout (*Oncorhynchus mykiss*)*

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2.1 Introduction

Viral haemorrhagic septicaemia is a disease of salmonid fish of great economic significance. The causative agent is the viral haemorrhagic septicaemia virus (VHSV) whose evolution and biogeography is complex and changing rapidly [1]. The virus is an enveloped negative single-stranded RNA virus that is part of the *Novirhabdovirus* genus of the *Rhabdoviridae* family with a genome encoding six viral proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonstructural protein (NV), and RNA-dependent RNA polymerase (L) [2]. Phylogenetic analysis of the N and G genes showed that VHSV can be categorized into genotypes-I, -II, -III, and -IV, with further sub-division into subgroups; for example “a”, “b”, “c”, “d” and “e” for genotype -I, and “a” and “b” for genotype-IV [3,4]. Perhaps most is known about genotype-Ia but interest in -IVa and -IVb has been quickly growing because they represent a new VHSV biogeography. These genotypes were first isolated from different geographical locations and at different times over the last 50 years showing different grades of virulence to rainbow trout (*Oncorhynchus mykiss*), making them good candidates for comparative studies of virulence. VHSV-Ia was first isolated in 1962 from a European rainbow trout freshwater aquatic facility and is highly virulent to this species [3]. VHSV-IVa was first isolated in 1988 from returning Chinook (*Oncorhynchus tshawytscha*) [46] and coho salmon (*Oncorhynchus kisutch*) [47]; the virus is highly virulent toward Pacific herring (*Clupea pallasii*) [5], but has a relatively low pathogenicity toward rainbow trout [6,31,33]. VHSV-IVb appeared first in 2003 in the North American great lakes, and despite having caused fish kills in many species [4,7,8], is only slightly pathogenic for rainbow trout [9, 30].

Although fin bases are capable of supporting VHSV replication and might be the most important site of *Novirhabdovirus* entry [10,11], the gill epithelium and macrophages are other early cellular targets that might contribute to viral pathogenesis in rainbow trout [13, 39]. Viral antigens were detected in the gill epithelium and in spleen macrophages relatively early upon infection of rainbow trout with a VHSV-Ia isolate [9,12] . Virulent, but not avirulent, isolates of genotype -I infected primary cell cultures of the rainbow trout gill epithelium [13] and replicated in rainbow trout gill fragments in organ cultures [14,29]. Although VHSV-Ia lysed primary kidney macrophage and leukocyte cultures [34,35], it failed to kill the rainbow trout monocyte/macrophage cell line, RTS11; VHSV-Ia expressed mRNAs for two viral genes, N and G, and one viral protein, N, in this cell line [15]. This investigation examines whether or not such

aborted infections would occur with the less virulent VHSV-IVa and -IVb genotypes in epithelial cells as well as in macrophages using the rainbow trout gill epithelial cell line, RTgill-W1, and the rainbow trout monocyte/macrophage cell line, RTS11. We have also compared the induction of genes related to the innate antiviral immune response in both cell lines in response to the different viruses. These include different type I interferon (IFN) genes, different Mx genes induced by type I IFN and the MDA5 and LGP2A receptor genes, known to activate the IFN upon recognition of viral RNA pathogen-associated molecular patterns in the cytoplasm.

RTgill-W1 supported the viral life cycle and produced viable VHSV-IVa and –IVb virions. By contrast, mRNAs for all six viral genes for both genotypes were expressed in RTS11 but no virions were produced and the cells remained viable. These results suggest that independent of the VHSV genotype the overall response of epithelial cells and macrophages is fundamentally different. However, subtle differences in the responses to VHSV-IVa and -IVb were seen with both cell lines. These consisted of differences in the mRNA expression levels for four viral genes and in the transcription induction for various antiviral genes.

2.2 Materials and Methods

2.2.1 Propagation of cell and virus

Three fish cell lines were used in this report: the recently reclassified fathead minnow EPC (*epithelioma papulosum cyprini*) cell line, and the rainbow trout gill (RTgill-W1) and monocyte/macrophage cell lines (RTS11) [16-18, 25]. EPC and RTgill-W1 were grown in 75 cm^2 tissue culture (TC) treated flasks (BD Biosciences) using Leibovitz's L15 medium (Hyclone, Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Fisher Scientific) and 1% penicillin-streptomycin (PS) (Hyclone, Fisher Scientific). RTS11 was grown in 25 cm^2 TC treated flasks (BD Biosciences) using L15 medium containing 15% FBS and 1% PS. All cell lines were grown at room temperature. The viruses used in this study were VHSV genotype IVb, isolate U13653 [8] and a North American VHSV genotype IVa isolate from Pacific herring [5]. Both isolates were propagated at 14 ^oC in confluent monolayer of EPC in 75 cm^2 TC treated flasks. Supernatants of infected cultures were collected from 7-10 days PI, centrifuged at 4444x*g* for 5 min to remove cellular debris, syringe filtered through a 0.2 micrometer filter (Pall Corporation) and stored at -80^oC. All viral titres were performed on EPC in 96-well TC treated plates (BD Biosciences) and calculated using the Karber method and expressed as TC infectious dose ($TCID_{50}$/mL) [19].

2.2.2 VHSV infection of cell cultures

Preparation of RTgill-W1 and RTS11 for infection trials was performed differently due to differences in the adherence properties of the two cell lines; RTgill-W1 is adherent and forms a monolayer, while RTS11 grows in suspension. RTgill-W1 was seeded at approximately four million cells in 25 cm^2 tissue culture (TC) flasks (Corning) in 4 mL of 10% FBS/L15 and allowed to attach overnight. The following day, old medium was removed and 4 mL of either control medium (2% FBS/L15), VHSV-IVb or –IVa medium were added; the final titre of virus in infected flasks was $1x10^7$ $TCID_{50}$/mL in 2% FBS/L15. RTS11 was seeded at approximately two million cells per well in 25 cm^2 TC flasks with either 4 mL of control medium (2% FBS/L15), VHSV-IVb or –IVa containing medium; the final titre of virus in infected flasks was $1x10^7$ $TCID_{50}$/mL in 2% FBS/L15. The flasks were incubated at 14^oC and monitored regularly for cytopathic effect.

To obtain samples for virus titration, RTgill-W1 and RTS11 were infected with VHSV-IVb or –IVa (1 x 10^7 $TCID_{50}$/mL) at 14^{o}C for approximately 2 h. Old medium was then removed and the cells were washed three times with 2 mL of D-PBS. For RTS11, cells were pelleted at 440xg for 5 min during each wash with DPBS; the wells that the cells were collected from were also washed three times. After washing, fresh 2 mL of 2% FBS/L15 was added to each well containing RTgill-W1, and in the case of RTS11, cells were resuspended in fresh 2 mL of 2% FBS/L15 and transferred to the wells. Immediately after, for the day zero time point, 100 µL was collected from each of the wells and stored at -80^{o}C for titration at a later time. Fresh 100 µL of 2% FBS/L15 was subsequently added and the plate was incubated at 14^{o}C for up to 8 days. Samples for day 4, and 8 were also collected in a similar manner.

2.2.3 Measurement of cell viability

RTgill-W1 was seeded at approximately 200,000 cells per well in 500 µL of 10% FBS/L15 into 24-well plates and allowed to attach overnight. The following day, old medium was removed and VHSV-IVb (diluted to 1x10^7, 1x10^6, 1x10^5, 1x10^4, and 1x10^3 $TCID_{50}$/mL), and VHSV-IVa (diluted to 1x10^8, 1x10^7, 1x10^6, 1x10^5, and 1x10^4 $TCID_{50}$/mL) in 500 µL of 2% FBS/L15 was added to separate wells, while 500 µL of 2% FBS/L15 containing no virus was added to parallel mock-infected control wells. The plates were incubated at 14^{o}C for up to 14 days. At time points of day 1, 4, 8 and 14, individual plates corresponding to each of those time points were measured for cell viability using the alamarBlue (AB) assay (Invitrogen); performed as previously described [20]. All RTgill-W1 cell viability experiments were performed in quadruplicate. RTS11 was seeded at approximately 600,000 cells per well in 900 µL of 2% FBS/L15 into 12-well plates; immediately after 100 µL of either VHSV-IVb or -IVa stock at 1x10^8 $TCID_{50}$/mL was added to experimental wells, making a final titre of 1x10^7 $TCID_{50}$/mL while, 100 µL of 2% FBS/L15 was added to the mock infected control wells. The plates were incubated at 14^{o}C for 1 h. Afterward, for the day zero time point, the suspended cells in day zero wells were mixed and 10 µL was removed and used to measure cell viability with the Countess Cell counter (Invitrogen) and trypan blue (Invitrogen) assay according to the manufacturer's instructions. Cell viability measurements for day 4, 8 and 14 were done in a similar manner and all RTS11 viability experiments were performed in triplicate.

2.2.4. Detection of viral proteins

RTgill-W1 and RTS11 cells infected with either control, VHSV-IVb or –IVa medium were collected, lysed in RIPA buffer [21] and frozen at -80^{o}C. Western blotting was performed according to the previously described protocol [21]. The primary antibody used was the mouse monoclonal antibody for VHSV N protein (1:600 dilution) [15]. Goat anti-Mouse IgG (H+L)-HRP conjugate (Bio-Rad) was used as the secondary antibody (1:5,000 dilution). Both primary and secondary antibodies were incubated either at room temperature for 1 h or overnight at 4^{o}C. Chemiluminescence was detected using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) in combination with a MF-ChemiBIS imager (DNR Bio-Imaging Systems).

2.2.5 Detection of viral transcripts by semi-quantitative RT-PCR and immune transcripts by real-time PCR

Total RNA was extracted from RTS11 and RTgill-W1 using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), and first strand cDNA synthesis was performed on approximately 2 µg of total RNA using the RevertAid H Minus Reverse Transcriptase kit with oligoDT primers (Fermentas, Thermo Scientific) following the manufacture's protocol. Viral gene amplification, using *Taq* DNA polymerase (recombinant) (Fermentas, Thermo Scientific), was performed with the primers listed in Table 2.1. The cycling conditions after initial denaturation at 95^{o}C for 3 min were as follows: denaturation at 94^{o}C for 1 min, primer annealing for 45 s, and elongation at 72^{o}C for 45 s. After the last cycle, there was a final extension step at 72^{o}C for 7 min. For all of the primers, the PCR was run for 35 cycles. PCR products were electrophoresed on a 1% agarose gel and subsequently imaged. Semi-quantitative RT-PCR analysis was done on results from three independent experiments. The analysis was performed using ImageJ [45]. The percentage intensities of each band in a row were measured for each gene and fold expression was normalized to the expression of a reference gene, β-actin [22].

For RT-qPCR, total RNA was extracted from RTS11 or RTgill-W1 cells using the PureLink® RNA Mini Kit (Invitrogen) following the manufacturer´s instructions. RNAs were treated with DNAse I (Invitrogen) to remove any genomic DNA traces that might interfere with the PCR reactions. One µg of RNA was used to synthesize cDNA in each sample using the Superscript III reverse transcriptase (Invitrogen) following the manufacturer´s instructions. The

resulting cDNA was diluted in a 1:4 proportion with water and stored at -20 ºC. To evaluate the levels of transcription of different immune genes, real-time PCR was performed with an Mx3005P^{TM} QPCR instrument (Stratagene) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures containing 10 µl of 2x SYBR Green supermix, 5 µl of primers (0.6 mM each) and 5 µl of cDNA template were incubated for 10 min at 95ºC, followed by 40 amplification cycles (30 s at 95ºC and 1 min at 60ºC) and a dissociation cycle (30 s at 95ºC, 1 min 60ºC and 30 s at 95ºC). Negative controls with no template were always included in the reactions. For each mRNA, gene expression was corrected by the elongation factor 1α (EF-1α) expression in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the EF-1α Ct value from the target Ct as previously described [22-24]. All the primers used had been optimized in previous studies and had been published elsewhere [22-24, 40]. All real-time PCR were performed in duplicate and two independent experiments were performed. Data handling, analyses and graphic representation was performed using Microsoft Office Excel 2003. Statistical differences were determined using a Student's *t* test.

Table 2.1 List of primers used in RT-PCR

Primer Names	Primer Sequences	Amplicon Sizes (bp)	References/ Accession Numbers (GenBank)
β-actin	F 5’ ATCGTGGGGCGCCCCAGGCACC 3’ R 5’ CTCCTTAATGTCACGCACGATTTC 3’	514	[44]
VHSV -IV-N	F 5’ AGGACCCCAGACTGTGCAAGC 3’ F 5’ TCCGCCTGGCTGACTCAACA 3’	605	DQ427105
VHSV -IV-P	F 5’ AGGAGAACGGGAAGAAGACCGACA 3’ F 5’ TCCAACTCCGCCTTGATTGCCT 3’	310	GQ385941
VHSV -IV-M	F 5’ TCAACCATCCTGACGGAAGGCA 3’ F 5’ TGATCAGGGTTTTGCTCCGGGT 3’	217	GQ385941
VHSV -IV-G	F 5’ CCCCGAACCTTCCTGCATCTGG 3’ F 5’ CCCGTCAGTGTTGTTGTCTATCCG 3’	739	DQ401195
VHSV -IV-Nv	F 5’ ACCCAAGCAACTACCTCAACTGTGA 3’ F 5’ AGAGTCCAGGATCCGACGGTCT 3’	228	GQ385941
VHSV -IV-L	F 5’ TGAGAGTGGGACAAGAAAGCTGGGA 3’ F 5’ GCACACGCTGTCCATCCTTGTCA 3’	511	GQ385941

Full name for each VHSV genes: N – nucleocapsid protein, P – phosphoprotein, M – matrix protein, G – glycoprotein, Nv – non-virion protein, L – RNA Dependent RNA polymerase.

2.3 Results

2.3.1. Observations of Cytopathic effects (CPE)

To determine whether VHSV-IVb and -IVa can cause CPE in rainbow trout macrophage and epithelial cells, RTS11 and RTgill-W1 were infected with high titre VHSV (~$1x10^7$ $TCID_{50}$/mL) isolates from both genotypes and continuously monitored for cellular alterations. As the RTS11 cell line mostly grows in suspension, researched CPE consists of cell morphology alterations; however, after 8 days of continuous exposure to VHSV-IVb and –IVa (Fig. 2.1b, c), RTS11 morphology did not change noticeably from mock-infected control cells (Fig. 2.1a). RTgill-W1 monolayer infected with VHSV-IVb resulted in partial destruction of the monolayer 8 days PI, with the majority of cells remaining viable (Fig. 2.1e). In contrast, VHSV-IVa almost completely lysed RTgill-W1 monolayer by 8 days PI, leaving few viable cells remaining (Fig. 2.1f). The monolayer of mock-infected control RTgill-W1 monolayer remained fully intact (Fig. 2.1d).

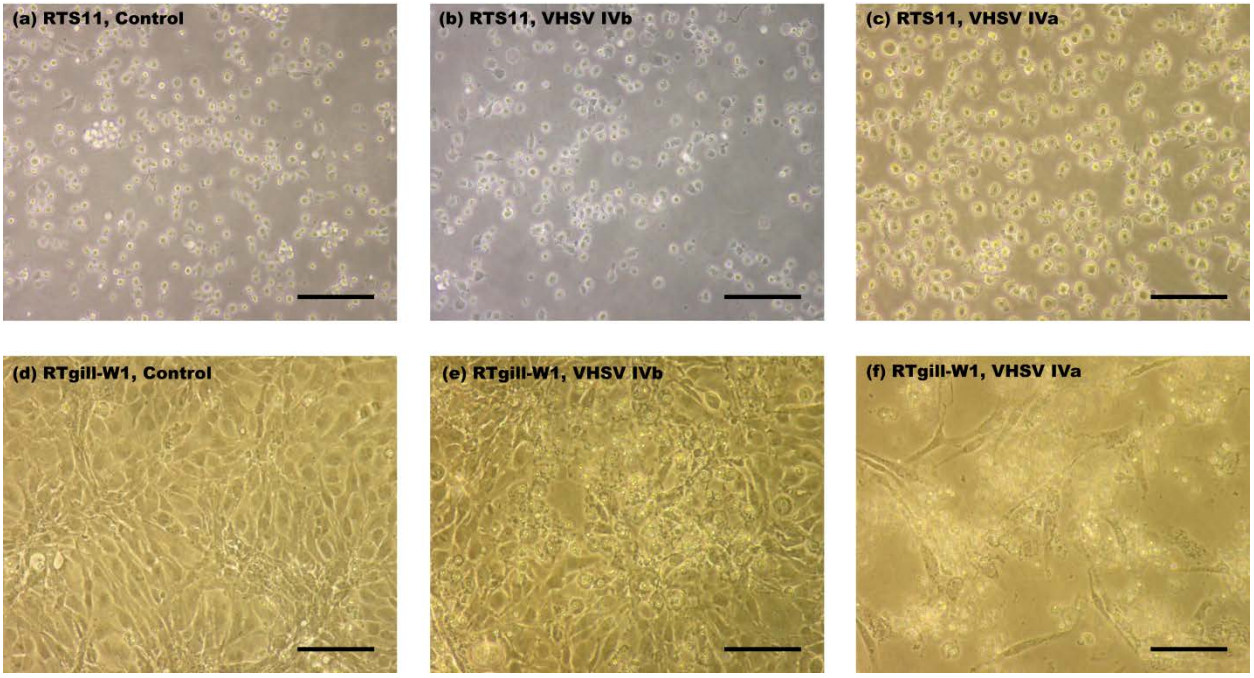

Fig. 2.1 Observation of cytopathic effects (CPE) in RTS11 and RTgill-W1

RTS11 infected with VHSV-IVb and -IVa showed no CPE (b, c, respectively) after 8 days of incubation with the virus; the cell morphology of infected cells is similar to that of control cells (a). RTgill-W1 infected with VHSV-IVb led to moderate destruction of the cell monolayer 8 days PI, resulting in floating debris (e), while VHSV-IVa almost completely destroyed the monolayer (f). In contrast, the monolayer of mock infected RTgill-W1 cells remained intact over the same period (d). The scale bar represents 100 μm.

2.3.2 Measurements of RTS11 and RTgill-W1 cell viability

Inasmuch as VHSV-IVb and -IVa failed to cause noticeable CPE in RTS11 but caused mild or near complete destruction of RTgill-W1 monolayer, respectively, the effects of these two genotypes on the viability of RTS11 and RTgill-W1 were quantified over a range of viral titres. Due to the different morphology of these cell lines, the effects of VHSV-IVb and -IVa on viability were quantified using two different techniques: trypan blue exclusion assay to measure membrane integrity of RTS11 and AB assay to measure metabolic activity of RTgill-W1 cells. After RTS11 was exposed to both VHSV isolates (~$1x10^7$ $TCID_{50}$/mL), the number of viable cells was counted over 14 days (at day 0, 4, 8, and 14); viable cell numbers did not decrease when infected with either isolate over that time course (Fig. 2.2a, b). This result supports our visual observations (Fig. 2.1b, c) that VHSV-IVb and –IVa do not cause CPE in RTS11. When RTgill-W1 was exposed to a range of infecting viral titres, a VHSV-IVb titre of $2.5x10^6$ $TCID_{50}$/mL and higher was required to cause cell death, whereas with VHSV-IVa, the lowest examined titre of $1x10^4$ $TCID_{50}$/mL reduced cell viability by day 8 (Fig. 2.2c, d). Cell death also occurred slower for VHSV-IVb infection than VHSV-IVa infection; RTgill-W1 cell viability was unaffected after 4 days at approximately $1x10^7$ $TCID_{50}$/mL of VHSV-IVb but reduced their viability to only 20% after the same exposure to VHSV-IVa (Fig. 2.2c, d). Therefore, VHSV-IVa can cause earlier destruction of RTgill-W1 monolayer, and the threshold titre required for near complete destruction is at least 2 logs lower than VHSV-IVb.

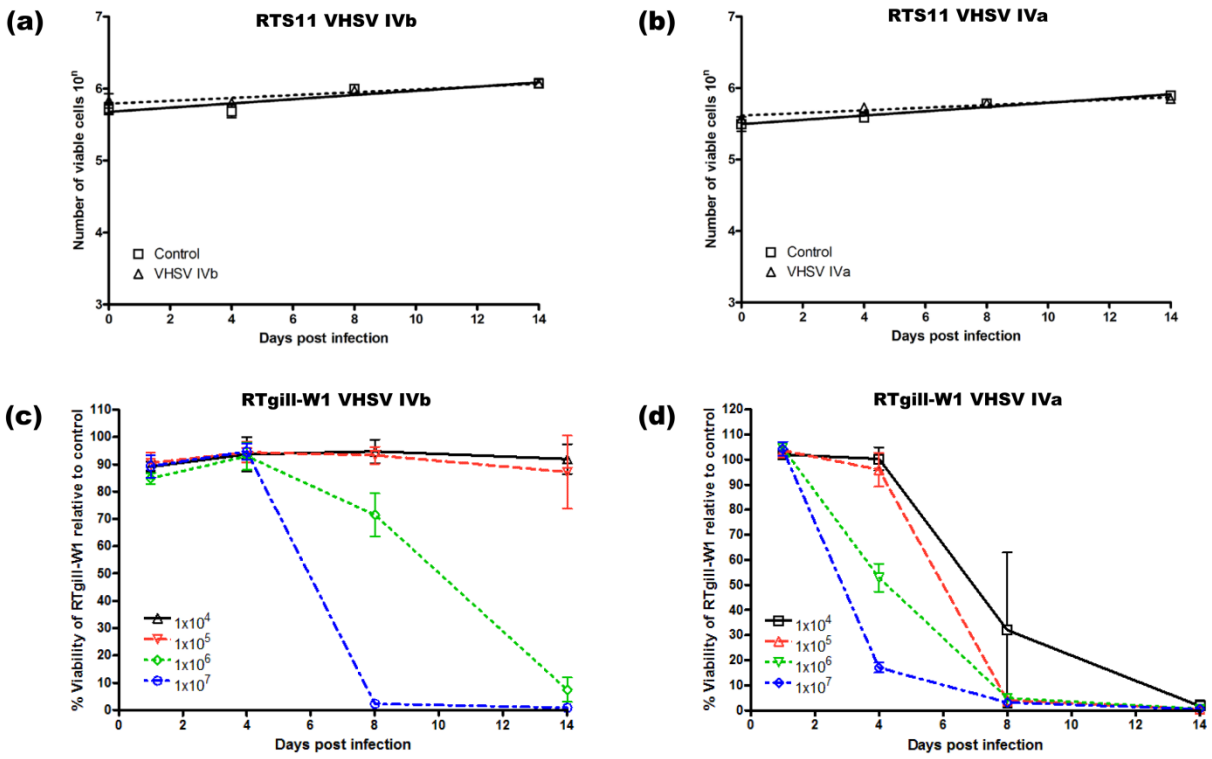

Fig. 2.2 Cell viability of RTS11 and RTgill-W1 infected with VHSV-IVb and -IVa

RTS11 was infected with either VHSV-IVb (a) or VHSV-IVa (b) and the cell viability was measured over 14 days. RTgill-W1 was infected with a titre range of either VHSV-IVb (c) or VHSV-IVa (d) and the cell viability was measured over 14 days. Data points for RTS11 and RTgill-W1 shows n = 3 ± SD and n = 4 ± SD, respectively.

2.3.3 Measurements of viral titre produced by RTS11 and RTgill-W1

When RTS11 and RTgill-W1 were infected with high titre VHSV-IVb and -IVa, the effect on cell viability was opposite; RTS11 survived while RTgill-W1 cells were lysed. To determine whether these two cell lines can support the production of viral progeny, they were infected with VHSV-IVb and -IVa for 2 h, cells were then washed and fresh medium was added to stop the exposition to virus. Over 8 days, the titre of VHSV-IVb and -IVa in the supernatant of RTS11 cells did not change from the residual amount that remained after washing on day zero. In contrast, viral titre in RTgill-W1 supernatant steadily increased over time to reach, at day 8, an average of $1x10^6$ $TCID_{50}$/mL for VHSV-IVb and $4x10^8$ $TCID_{50}$/mL for VHSV-IVa (Fig. 2.3). VHSV-IVa produced an average of 1.6 logs higher titre than -IVb by day 8. Therefore the death of RTgill-W1 is caused by the replication and production of viral progeny, whereas RTS11 is unable to produce infectious viral progeny.

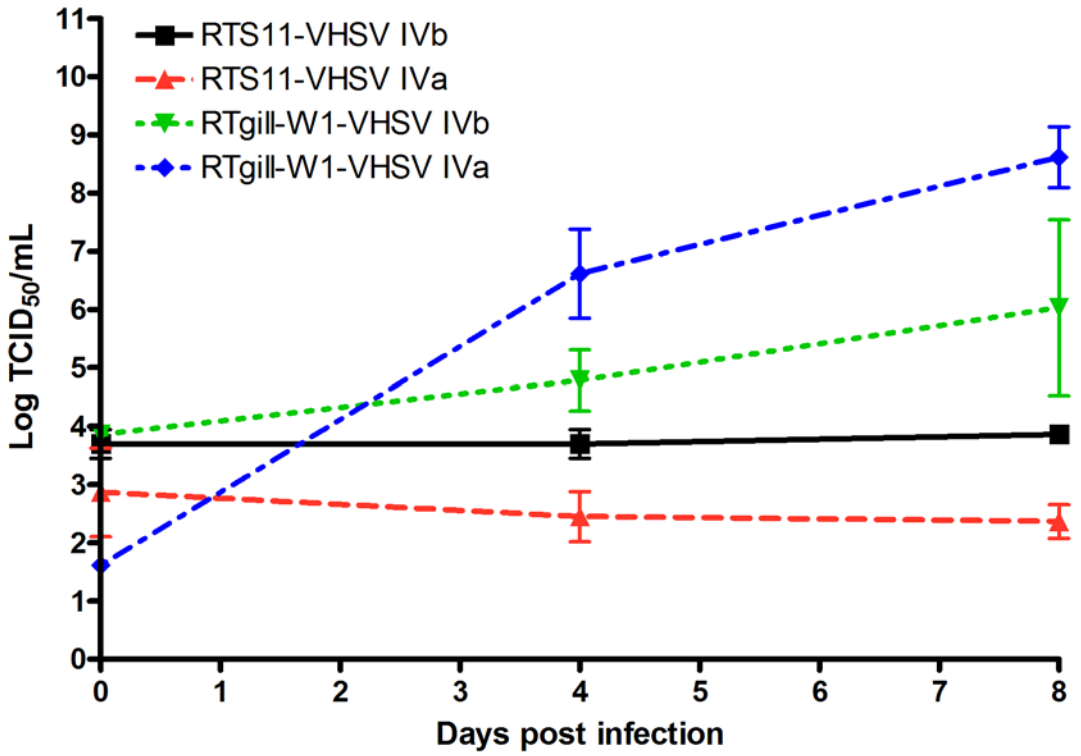

Fig. 2.3 Titre of VHSV-IVb and -IVa produced by RTS11 and RTgill-W1 infected cells

The amount of VHSV-IVb and -IVa in the supernatant of these cells was quantified at various time points over 8 days. There was no change in VHSV-IVb and -IVa titre in the supernatant of RTS11 over the 8 days whereas viral titre in RTgill-W1 supernatant increased over time. The titre of VHSV-IVb and -IVa was log transformed to show log changes. Data points represent n = 3 ± SD.

2.3.4. Determination of viral entry and gene expression

Although RTS11 was unable to produce infectious VHSV-IVb and -IVa progeny, the cause could either be due to the inability of the virus to enter RTS11, or to complete the viral replication cycle once it has entered. To determine whether VHSV-IVb and -IVa can enter RTS11 and express viral genes, RT-PCR using primers targeting conserved regions of all six viral genes was performed on total RNA of RTS11 infected cells collected at various time points. Transcripts from all VHSV-IVb and -IVa genes were detected as early as 1 day PI in both RTS11 and RTgill-W1 infected cultures (Fig. 2.4a). To compare the level of transcript expression of the six viral genes between VHSV-IVb and –IVa in RTS11 and RTgill-W1, semi-quantitative RT-PCR was used. The transcript level of each VHSV gene was compared at day 1 PI for RTS11 and RTgill-W1. In both RTS11 and RTgill-W1, VHSV-IVa expressed higher transcript level of viral N, P, G, and L genes than VHSV-IVb at day 1 PI (Fig. 2.4b). Interestingly, the transcript level of VHSV-IVb and -IVa M and NV genes remained consistently similar to each other in both RTS11 and RTgill-W1 (Fig. 2.4b). When protein from RTS11 infected cells were probed using a monoclonal antibody against viral N protein, no viral N protein was detected in both VHSV-IVb and –IVa infected RTS11 cultures, but the same antibody detected N protein in RTgill-W1 cells infected with VHSV-IVb and -IVa as early as day 4 PI (Fig. 2.4c). Therefore, these results suggest that although both VHSV-IVb and -IVa are capable of entering and expressing viral transcripts in RTS11, the infection from both isolates was aborted before the expression of viral protein occurred.

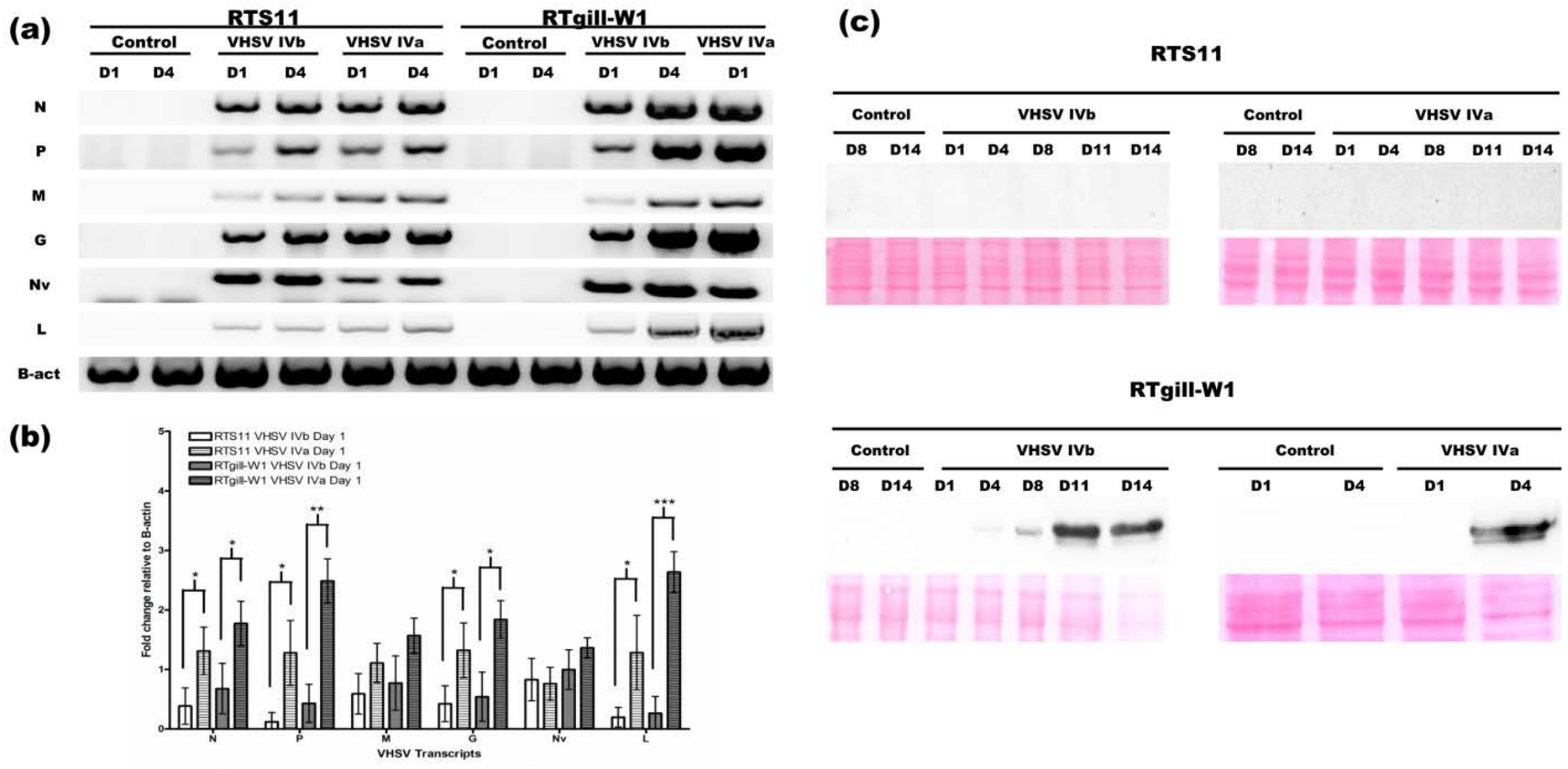

Fig. 2.4 Detection of VHSV transcripts in RTS11 and RTgill-W1, and VHSV N protein in RTgill-W1

(a) Representative image and (b) semi-quantitative RT-PCR graph of all six viral transcripts detected in RTS11 and RTgill-W1. B-acting (B-act) primers were used as loading control. For RTgill-W1 infected with VHSV-IVa, day 4 was not collected because the virus completely lysed the cell monolayer by that time point. Each bar on the graph shows n = 3 ± SD. Levels of statistical significance: * ($p < 0.5$), ** ($p < 0.01$), *** ($p < 0.001$). (c) Ponceau stain and corresponding western blot showing detection of viral N protein in RTgill-W1 infected with either VHSV-IVb or -IVa, but not in RTS11.

2.3.5. Determination of RTS11 and RTgill-W1 antiviral response to VHSV

Following the detection of VHSV transcripts in RTS11 we proceeded to investigate, using real time RT-PCR, whether a panel of innate antiviral response genes, Mx1, Mx2, Mx3, IFN1, IFN2, IFN3, MDA5 and LGP2A were regulated by the viruses in RTS11 and RTgill-W1 after 2 days of infection. RTS11 expressed detectable constitutive levels of Mx1, IFN1, IFN2 and LGP2A (Fig. 2.5a), whereas RTgill-W1 constitutively expressed Mx3 and IFN1 in control cells (Fig. 2.5b). RTS11 infection with both VHSV-IVb and –IVa resulted in increased transcript levels of Mx2 and Mx3 while Mx1, IFN1 and IFN2 showed no changes. IFN3 and MDA5 expression was not detectable and remained unchanged after viral infection. A faint LPG2A signal was detected in the control but did not appear in the virus infected cells, which may suggest a negative effect of the viruses on the levels of LPG2A mRNA. When RTgill-W1 was infected with either viral genotype, the expression of Mx2 and Mx3 was also significantly up-regulated as in RTS11 with similar fold-changes relative to control mock-infected cultures (Fig. 2.5b). In RTgill-W1, IFN1 was also significantly up regulated in response to both VHSV-IVa and –IVb, with approximate fold-changes relative to mock-infected cultures of 23292 and 64, respectively. IFN2 and LGP2A were only up regulated by VHSV-IVa in RTgill-W1, and not by VHSV-IVb, relative to control cells, with expression levels approximately 5873 and 463 fold higher than mock-infected cultures, respectively (Fig. 2.5b). Similar to RTS11, IFN3 and MDA5 was not detected at a significant level in RTgill-W1 (Fig. 2.5b).

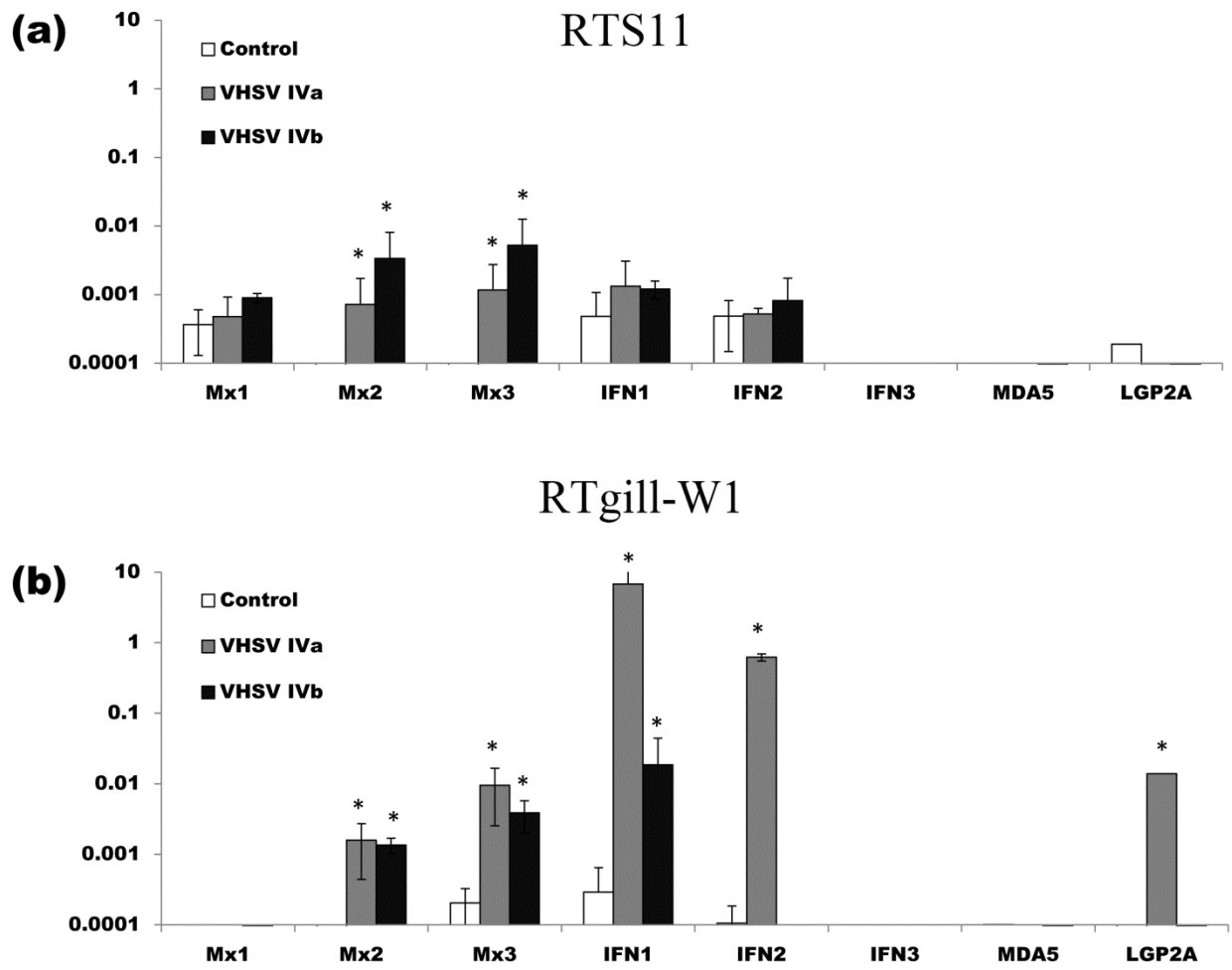

Fig. 2.5 RT-qPCR of immune genes detected in RTS11 and RTgill-W1 cells infected with VHSV-IVb and -IVa.

RTS11 (a) and RTgill-W1 (b) were infected with either control, VHSV-IVa or -IVb medium and examined for expression of a panel of innate immune genes. Each bar represents n = 2 ± SD. * Levels of relative transcription significantly different than those observed in mock-infected cultures ($p < 0.05$).

2.4 Discussion

In the current work, the responses of two rainbow trout cell lines, RTgill-W1 from the gill epithelium, and RTS11 from spleen macrophages, to infection by VHSV-IVa and –IVb were compared. The gill epithelial cell line was lysed by both viruses, expressed all six VHSV genes and produced infectious titre; however, VHSV-IVa appeared more infectious to RTgill-W1 cells than VHSV-IVb. The macrophage cell line expressed all viral genes of both strains but remained viable and failed to produce new viral progeny.

The results for RTgill-W1 with either VHSV-IVa or -IVb provide additional support for the gill epithelium being a site of VHSV replication [13-14,29], and thus possibly contributing to viral entry and exit. RTgill-W1 fully supported VHSV-IVb and -IVa replication as seen by the development of CPE, cell death and increase in viral titre. However, RTgill-W1 did not appear to be exceptionally susceptible. Relative to the fathead minnow cell line, EPC, which is commonly used to isolate genotype -I and -IV [5,25] but not genotype -II [26], RTgill-W1 was less sensitive. A higher VHSV-IVb titre of $2.5x10^6$ $TCID_{50}$/mL was required to establish cell death in RTgill-W1 cultures, whereas in EPC cultures significant cell death was seen at very low values ($1x10^2$ $TCID_{50}$/mL) [20]. Interestingly, RTgill-W1 has been used to isolate genotype -III from a VHS outbreak on a Norwegian rainbow trout farm [27], but whether genotypes -I and -II can be produced on RTgill-W1 has yet to be examined. In cultures of excised gill pieces, which can be considered organ cultures, VHSV genotype-Ic (23/75) replicated to high titre in gills from rainbow trout and Chinook salmon [14]. *In vivo*, the importance of the gill epithelium as a site of viral replication has been revealed through immunohistochemical studies on the localization of VHSV antigens in infected fish. Viral antigens were seen in the gill epithelium of rainbow trout infected with genotypes -Ia [12] and -IVb [9]. For rainbow trout challenged by waterborne exposure to VHSV-Ia, virus levels tended to be highest in pooled internal organs of clinically affected fish and in pooled gill and brain tissue samples of surviving fish [28]. Overall these observations point to the gill as an important organ in the development and recovery from viral hemorrhagic septicemia.

Studies with rainbow trout gill organ cultures [14,29], primary gill cell cultures [13], and now the cell line RTgill-W1 suggests a correlation between the ability to replicate *in vitro* and the virulence of different VHSV genotypes *in vivo*. When VHSV production was compared on excised gill pieces from VHSV resistant and susceptible rainbow trout, the virus titre was 2 logs

higher in gill cultures from susceptible than those from resistant fish [29]. Virulent VHSV strain –Ic (isolate 23/75) replicated to high titre in excised gill tissues whereas the less virulent North American Makah strain, which belongs to genotype -IVa, failed [14]. Similarly, a marine isolate of VHSV genotype -Ib showed significantly delayed translocation across an epithelial cell monolayer in primary rainbow trout gill cell cultures and was unable to reduce epithelial cell viability 7 days after exposure whereas a freshwater VHSV-Ia isolate translocated across epithelial cell monolayers in 2 h and significantly reduced cell viability by day 3 [13].

With RTgill-W1, strain -IVa caused CPE to develop more quickly and at lower titre than did -IVb. Although both genotypes are less pathogenic to rainbow trout than genotype -I [1], with the possible exception of one study with -IVb in young rainbow trout [30], generally -IVa has appeared more virulent than -IVb to adult rainbow trout. Mortality was as high as 12% with -IVa [31-33] but has never exceeded 4.4% with -IVb, regardless of whether waterborne or intraperitoneal exposures were tried [9]. However, due to the low mortalities of the above mentioned studies, the differences may not be true biological difference between VHSV –IVa and –IVb but could be attributed to the fact that the studies were conducted by different laboratory groups and different fish stocks were used. To determine actual biological difference, head-to-head challenge of a common rainbow trout stock with VHSV –IVa and –IVb must be done.

Although different from some early observations where leukocytes and macrophages supported VHSV replication [34,35], the inability of RTS11 to produce VHSV or to show a loss of viability was similar to recent rainbow trout studies that have detected VHSV infection in only a small percentage of macrophages exposed to the virus [13]. In the current work, RTS11 failed to support replication of genotypes -IVa and -IVb, and previously we demonstrated their inability to produce genotype -Ia [15]. Other studies have used rainbow trout primary cell cultures. When leukocytes were infected with genotype -I only about 8% was infected but the titre increased 2 logs, indicating that some viral replication occurred [36]. For head kidney macrophage cultures infected either with virulent freshwater or avirulent marine isolates of genotype -I, the majority of the cells remained viable and less than 10% of the cells were immunologically positive for viral antigen [13]. Spleen macrophages positive for viral antigen have been seen *in vivo* for rainbow trout challenged with -IVb [9]; however, these macrophages were enlarged and contained cell debris and therefore they might not necessarily be sustaining VHSV replication

but instead might be positive because they had engulfed dead cells that had supported viral infection. In summary, the lack of infection with RTS11 and low level of infection with primary macrophages might be due to the homogenous composition of the cell line when compared to *in vivo,* which contains mixed populations of macrophages and other stromal cells.

A remarkable feature of RTS11 was that while they failed to produce VHSV, they did support the expression of VHSV genes. Previously, we found that upon infection with genotype -Ia RTS11 cultures expressed two viral transcripts, N and G, as determined by RT-PCR, and one viral protein, N, but not G, was strongly detected in western blots [15]. VHSV-IVa and -IVb transcripts were detected in RTS11 for all six viral genes but N protein was not detected. Thus genotypes -Ia, -IVa and -IVb can enter RTS11 and begin a replication cycle that is subsequently aborted. The expression of transcripts for all six viral genes in RTS11 suggests that this abortion was not due to the lack of transcription of any single viral gene but due to the lack viral mRNA translation. An alternative but unlikely scenario is that rather than the viral life cycle being disrupted a few cells in RTS11 cultures support a complete viral life cycle and the number of cells doing this would be too small to see a change in viral titre and cell viability and the accumulation of viral protein.

Looking at the antiviral response genes expression in RTS11 and RTgill-W1 to VHSV-IVa and –IVb, there appears to be differential induction of antiviral genes between the two strains; this may be related to the virulence of these virus strains in rainbow trout, and some of these differences are consistent with previously published works. VHSV-Ia up regulated Mx1, IFN1 and IFN2 transcript level beyond that of control cells [15], but this was not the case for VHSV-IVb and –IVa in this study. The higher virulence of VHSV-Ia toward rainbow trout fish may account for this observed difference. A similar result was observed for the related IHNV virus in the RTG-2 cell line where the more virulent M-type IHNV induced higher transcript levels of IFN1 and Mx1 in RTG-2 cells than the less virulent U-type [37]. At whole fish level, the results are also consistent; a highly virulent isolate of genotype -Ia induced higher transcript levels of Mx1 and IFN1 in both immersion and intraperitoneal challenges of rainbow trout fry than a less virulent isolate [38], and M-type IHNV induced higher Mx1 transcript levels in the anterior kidney of rainbow trout and in whole juvenile rainbow trout than U-type IHNV [39]. For RTgill-W1 cultures during the first 2 days of infection, transcripts for Mx2, Mx3, and IFN1 were up-regulated by both VHSV-IVa and –IVb; however, VHSV-IVa up regulated IFN2 and LGP2A

while VHSV-IVb did not. Furthermore, no expression changes were detected for IFN3, MDA5 or LGP2A in RTS11, and no changes of IFN3 and MDA5 were found in RTgill-W1, by either VHSV-IVa or –IVb, while VHSV-Ia was previously reported to up regulate both MDA5 and LGP2A genes in RTS11 and RTG-2 cell lines [24]. Looking at these antiviral genes, similar to the case with Mx1 and IFN1, we noticed that the virus isolate that is more infectious appears to induce more antiviral genes. As an interesting aside, IFN3 was previously shown to be induced in organs such as head kidney, spleen, liver, and ovary, but it was not detected in cell lines such as RTS11 and RTG-2 [23,40]. Here, we confirm the lack of IFN3 detection in RTS11 and extend this finding to another cell line, RTgill-W1, thus corroborating that IFN3 is not expressed at detectable levels in either gill cells or gill tissues [40].

The cause of the aborted VHSV infection in RTS11 is open to discussion but we speculate that the antiviral Mx genes may play a role. VHSV consistently up regulated at least two antiviral genes, Mx2 and Mx3, in RTS11; similar results were observed after RTS11 was infected with VHSV-Ia for 2 days [15]. Interestingly, we noticed that RTS11 showed a constitutive level of Mx1 expression that is not present in RTgill-W1. Higher Mx1 expression could have helped terminate the VHSV life cycle in RTS11 because the antiviral activity of Mx has been previously demonstrated with fish rhabdoviruses. A Japanese flounder embryo cell line expressing transfected Mx cDNA against VHSV and hirame rhabdovirus, had reduced amounts of viral proteins and replication [41]. Whether aborted VHSV infections alter the immunological and physiological functions of macrophages is an interesting future question. Another interesting question is the occurrence of aborted infections *in vivo*. Visualizing viral RNA transcripts by *in situ* hybridization in fish tissues after infections might hint at an answer. Viral transcripts in many cell types unaccompanied by signs of cellular pathology and of clinical symptoms would suggest aborted infections.

The responses of the two rainbow trout cell lines to VHSV-IVa differed from the responses to VHSV-IVb in three ways: first, strain -IVa was more virulent than -IVb toward RTgill-W1; a higher titre of $2.5x10^6$ $TCID_{50}$/mL was required for VHSV-IVb to cause significant CPE in RTgill-W1 cultures, whereas for VHSV-IVa a titre of $1x10^4$ $TCID_{50}$/mL was enough. Secondly the levels of viral gene expression differed between the two isolates. For four of the viral genes, N, P, G and L, the transcript level of these genes in VHSV-IVa infected RTS11 and RTgill-W1 cells was consistently higher than in cell infected with VHSV-IVb. A similar result was observed

by Park et al. [42] with IHNV; avirulent U type IHNV produces 37-fold lower genome replication and 23-fold lower level of viral G transcripts after 12 hr PI in RTG-2 cell line when compared to the virulent M type. The difference(s) that contributes to these varying cellular responses are unknown but certain viral genes seem more likely to be important than others. Previous sequence comparison of four VHSV isolates of varying degrees of virulence toward rainbow trout identified only one amino acid substitution in each of the N, G, NV and L protein, suggesting that differences in virulence can be cause by a few amino acid changes [43]. For example, if VHSV-IVa were to have a more efficient viral RNA polymerase (L), this might lead to the more rapid accumulation of viral mRNAs and an earlier initiation of cell death in cultures. Thirdly, IFN1, IFN2 and LGP2A were up regulated in RTgill-W1 to higher levels by VHSV-IVa than –IVb, and this is in line with the previously discussed general pattern of more virulent VHSV and IHNV isolates inducing higher level of immune gene expression in susceptible cell line, organs and fish.

In conclusion, the gill epithelial cell line, RTgill-W1, supported the production of VHSV strains -IVa and -IVb, whereas the macrophage cell line, RTS11, did not. As with a previous study on genotype Ia, IVa and IVb had aborted infections in RTS11. The expression of mRNA for all six VHSV genes in infected RTS11 culture coupled with the absence of detectable viral N protein, lack of increase in viral titre, and no cell death were evidence of an aborted infection. With regards to immune response of host cells we noticed that the differential up regulation of Mx1 and IFN1 genes by VHSV isolates of varying virulence is similar to that described in previous work on VHSV and IHNV. However, differences in the ability of each cell line to express other innate immune genes in response to both VHSV-IVa and -IVb was also observed, and the difference further extends to observation of RTS11 and VHSV-Ia in previously published studies. These results point to the likely complexity of tissue responses in fish upon infection with VHSV and the necessity to examine different cell types and viral genotypes to build up a wider picture of viral pathogenesis.

CHAPTER 3

Differential viral propagation and induction of apoptosis by grouper iridovirus (GIV) in cell lines from three non-host species*

*This chapter has been published in the following article: Pham PH, Lai YS, Lee FF, Bols NC, Chiou PP. Differential viral propagation and induction of apoptosis by grouper iridovirus (GIV) in cell lines from three non-host species. Virus Res. 2012;167(1):16-25.

3.1 Introduction

The mechanisms underlying virus host range and host shifting in aquatic animals are poorly understood, but are important for understanding emerging infectious diseases in aquaculture [1,2]. To be a host, a species must be exposed to the virus and support replication of the virus; in host shifting, the range of species in which this can be done is expanded [3]. The restraints that define host ranges and influence host shifting likely occur at multiple levels of biological organization. At the cellular level, the virus must be able to enter host cells, replicate successfully, and exit from the cell [4]. The most common internalization route for animal viruses is receptor-mediated endocytosis, but new routes continue to be discovered, such as caveolin-lipid raft mediated pathway, macropinocytosis, and phagocytosis [5,6]. These different internalization routes deliver viruses to various cellular compartments, including acidic endocytic vacuoles, the endoplasmic reticulum, and the cytoplasm, resulting in different outcomes [6]. One outcome is cell death accompanied by the release of virions. Cell death can occur in different ways, with apoptosis being most common. Apoptosis is a programmed cell death characterized by features such as genomic DNA fragmentation and phosphatidylserine externalization [7].

Ranaviruses are large, double-stranded DNA viruses of the family *Iridoviridae* with broad host ranges and possible host shifts [1,2]. An example is epizootic hematopoietic necrosis virus (EHNV). EHNV was isolated in Australia and found to infect a wide range of fish, including aquaculture species like rainbow trout [8]. The evolutionary history of EHNV appears to contain multiple host shifts, possibly including jumps from fish to frogs, from fish to salamanders, and from frogs to reptiles [2]. Also placed among the *Ranaviruses*, but tentatively, are the grouper-like *Ranaviruses* [2,9]. Grouper iridovirus (GIV) was isolated from diseased yellow grouper, *Epinephelus awoara* [10]; Singapore grouper iridovirus (SGIV), from diseased brown-spotted grouper, *Epinephelus tauvina* [11]. Both viruses have been shown to be significant pathogens for these fish [11,12], which are valuable commodities in Asia and subject to exportation [13]. *Ranaviruses* are possibly moving around the world in host species marketed as bait, food, pets, and research animals [2,14,15]. Thus the potential exists for new *Ranavirus* hosts and for the emergence of new *Ranavirus* pathogens.

Cell lines from different species and tissues can be used to evaluate the cellular basis of host restrictions and potential for host shifting. Therefore, we have studied the ability of GIV to

infect the cell lines, RTS11, CHSE-214, and EPC from respectively rainbow trout (*Oncorhynchus mykiss*), which is a prominent species for aquaculture, Chinook salmon (*Oncorhynchus tshawytsch*), which supports a fishery, and fathead minnow (*Pimephales promelas*), which is commonly used as bait. RTS11 is a monocyte/macrophage cell line that is phagocytic [16]; CHSE-214 is an embryonic epithelial cell line with an impaired capacity for endocytosis [17]; and EPC is a skin epithelial cell line [18], presumably with a normal capacity for endocytosis. Despite the cell lines having different functional capabilities and species and tissue origins, GIV was able to enter each cell line and induce apoptosis, but only EPC supported the propagation of GIV.

3.2 Materials and Methods

3.2.1 Cell culture

Four cell lines were used in this study: grouper kidney (GK) [10], rainbow trout monocyte/macrophage RTS11 [16], Chinook salmon embryo (CHSE-214) and *Epithelioma papulosum cyprini* (EPC). CHSE-214 and EPC can both be obtained from the American Type Culture Collection (ATCC). Current linage of EPC, initially thought to have been from carp has been recently reclassified as a fathead minnow cell line [19]. All cell lines were grown in L15 medium supplemented with fetal bovine serum (FBS, 10% for GK, CHSE-214, and EPC, and 15% for RTS11). For RTS11, a ratio of 1:4 of conditioned medium from previous passage to fresh medium was used when passaging. GK was grown at 28 ^{o}C while CHSE-214, EPC and RTS11 were grown at 20 ^{o}C on Corning 75-cm^2 tissue culture flasks. GK, CHSE-214 and EPC were passaged approximately once every 4-5 days while RTS11 were passaged once every 7-8 days, at a ratio of 1:4 for GK and EPC, 1:2 for CHSE-214 and RTS11.

3.2.2 Virus propagation and treatment with heat and UV

Grouper iridovirus (GIV) [10] was propagated on GK cells in 75-cm^2 flasks at 28 ^{o}C in L15 with 2% FBS. The virus and cell debris were collected 7 days post infection. The mixture was centrifuged at 1000 xg for 5 min to pellet cellular debris. The supernatant was then suction or syringe filtered through a 0.2-micrometer filter from Corning Inc. Collected GIV was separated into aliquots and frozen at -80^{o}C until use. All viral titres in this report were calculated as tissue culture infectious dose ($TCID_{50}$/mL) using the Karber method [20]. For certain experiments, GIV was either heat- or UV-treated separately, after thawing, before infecting cell lines. In the heat treatment, GIV was incubated at 60 ^{o}C for 2 h. In the UV treatment, GIV was seeded on 60-mm tissue culture dish (Corning Inc.) and was exposed to 150 mJ of energy in a UV Stratalinker ® 2400 (Stratagene).

3.2.3 Infection of cell lines with GIV for cytopathic effects observation and apoptosis assays

The cell lines, GK, RTS11, CHSE-214, and EPC, were seeded in wells of a 6-well plate at approximately 1.0 x 10^6 cells/well in L15 medium with 10% FBS for GK, CHSE-214, and EPC, and 15% FBS for RTS11. The plates were incubated overnight at the growth temperature for each cell line for use the next day. Untreated stock GIV, along with heat- and UV-treated GIV, with titre of approximately 2.49 x 10^8 $TCID_{50}$/mL, was diluted 100 fold in L15 with 2% FBS to make final titre of 10^6 $TCID_{50}$/mL. To each of the seeded wells, 2 mL of the diluted virus was added on the monolayer of cells after the old medium was removed, making a final multiplicity of infection of 1. For each virus treatment two replicates were performed. The plates were then incubated at 20 ^oC overnight (12 h) and observed (with pictures taken) and assayed the next day for apoptosis by DNA laddering and Annexin V Staining.

3.2.4 Identification of apoptosis by DNA laddering and Annexin V staining

The two methods used to identify apoptosis in cell lines are DNA laddering and Annexin V staining. For DNA laddering, the protocol is performed as follows. After being infected with GIV, cells were detached from 6-well plates using trypsin and pelleted at 1,000 xg for 5 min. Pelleted cells were lysed with lysis buffer (0.2% v/v Triton X-100, 10 mM Tris pH 8.0, and 1 mM EDTA) and incubated on ice for 10 to 15 min. The lysate was then centrifuged at 13,000 xg for 15 min at 4 ^oC. Following centrifugation, the supernatant was kept and incubated for 1 h with RNase A (final concentration, fc: 65 µg /mL) (US Biological), followed by addition of SDS (fc: 0.5% v/v) and proteinase K (fc: 160 µg/mL) (Invitrogen). The mixture was then incubated at 50 ^oC for 1 h. After incubation, NaCl (fc: 0.45M) was added, followed by precipitation in 1 volume of ice-cold isopropanol for 15 min. The precipitated DNA was pelleted at 13,000 xg for 15 min at 4 ^oC, washed with 75% ethanol and resuspended in TE buffer. The DNA samples were run on a 1.8% agarose gel. The detection of phosphatidylserine, an early marker of apoptosis, was done using the PE Annexin V Apoptosis Detection Kit I from BD Biosciences. Cells were stained with PE Annexin V and 7-AAD according to the manufacturer's protocol. Cells that are stained with only PE are in early apoptosis and with only 7-AAD, necrosis. Cells that are stained with both cannot be differentiated between late apoptosis and necrosis; and, cells that do not stain with either are viable. Detection and quantification of apoptotic cells were conducted using a Gallios Flow Cytometer and accessory software from Beckman Coulter. Statistical significance was

determined by one-way analysis of variance (ANOVA, $p<0.05$), followed by Bonferroni Multiple Comparison test.

3.2.5 Detection of viral gene expression

Cell lines, GK, RTS11, CHSE-214, and EPC were seeded in 6-well plates and infected with GIV as described in the previous section; similar procedures were also done for the controls. After washing with buffered saline, and addition of fresh medium, the plates were incubated at 20 ^{o}C. At 1 h, 6 h, and 12 h PI, the cells in each individual control and virus exposed wells (each time point represents separate wells), were collected using trypsin. Cells were pelleted at 500 xg for 5 min, followed by removal of the old medium and washing with buffered saline. Cells were then pelleted a second time at the same speed and length of time before the wash solution was removed and the pellet immediately frozen at -80 ^{o}C for processing at a later time. Once all samples were collected, the pellets were thawed and total RNA was extracted using TRIzol Reagent from Invitrogen following the manufacturer's protocol. Following initial extraction, the RNA was incubated at 37 ^{o}C for 30 min with DNase (RQ1, Promega). The RNA was then subjected to the TRIzol extraction process for a second time to ensure removal of DNase and any potential contaminating DNA. The RNA was then reverse-transcribed into cDNA using Superscript III Reverse Transcriptase from Invitrogen under the following temperature conditions: 1) 25 ^{o}C for 5 min, 2) 42 ^{o}C for 50 min, and 3) 95 ^{o}C for 5 min. Polymerase chain reaction, using Pro Taq (Protech Technology, Taiwan), was done using the primers listed in Table 3.1 according to the following cycling conditions: 95^{o}C for 3 min, (94 ^{o}C for 1 min, X ^{o}C for 1 min, 72 ^{o}C for 1 min) x 35 cycles, and 72 ^{o}C for 7 min.

Table 3.1 List of primers, annealing temperature and cycle numbers used in RT-PCR

Primer Names	Primer Sequences	Annealing Temp. (X)	References
β-act-RTS11	F: ATCGTGGGGCGCCCCAGGCACC R: CTCCTTAATGTCACGCACGATTTC	53^{o}C	[35]
β-act-CHSE	F: ATTCGCCGGAGATGACGCGC R: CAGCGCCACGTAGCACAGCT	55^{o}C	
β-act-GK	F: GATCATGTTCGAGACCTTCAACAC R: TCCAATCCAGACAGAGTATTTACG	54^{o}C	
β-act-EPC	F: CCGAGGCTCTCTTCCAGCCAT R: GGTGGAAGGAGCAAGGGAGGTG	55^{o}C	
GIV MCP	F: CGCGGATCCATGACTTGTACAACGGGT R: CCGCTCGAGCAAGATAGGGAACCCCAT	55^{o}C	
GIV 030L	F: CGCGGATCCATGCTGTTTGTAGCG R: CCGCTCGAGATCCATTTTGCGGTT	55^{o}C	
GIV 097L	F: CGCGGATCCATGGAATGTTTATAC R: CCCAAGCTTCACAAACCCAAATTT	55^{o}C	

MCP, 030L, and 097L are primers targeting the genes encoding the major capsid protein, TNFR domain, and predicted E3 ubiquitin ligase domain of GIV, respectively.

3.2.6 Determining viral replication in cell lines infected with GIV

Cell lines, GK, RTS11, CHSE-214, and EPC were seeded in 6-well plates and incubated overnight as previously described. The next day, old medium was removed and 200 µL of stock GIV at 2.49 x 10^8 $TCID_{50}$/mL was added to 2 duplicate wells for each cell line; for control wells, 200 µL L15 with 2% FBS was added instead. The solutions were evenly dispersed over the monolayers and the plates were incubated at room temperature for 1 h to allow for viral entry into cells. After 1 h, the wells were washed twice with buffered saline, followed by addition of 2 mL of fresh L15 with 2% FBS. At this point, 150 µL was removed from each well and saved for titration at time point day zero. Immediately after the removal, 150 µL of fresh L15 with 2% FBS was added to each well and the plates were incubated at 20 ^oC for RTS11, CHSE-214 and EPC, and 28 ^oC for GK cells. Titration was done on the same day on confluent monolayer of GK cells in 96-well plates seeded the night before, and performed by serial dilution using the tissue culture infectious dose method. Samples were also taken at day 3 and day 7, and titrated in a similar manner. Two replicates were performed for each cell line and time point. For graphical representation and statistical analysis, the $TCID_{50}$/mL value of each time point were log transformed using the following formula: log ($TCID_{50}$/mL). Linear regression were performed on the log-transformed values for all three cell lines and time points to determine if any increase in titre over the 7 days is significant. Log changes in titre relative to day zero were calculated as follows: log ($TCID_{50}$/mL of Day X / $TCID_{50}$/mL of Day zero), where X is either 3 or 7.

3.2.7 Electron microscopic observation of EPC cells infected with GIV

Monolayers of EPC cells were infected with GIV with a M.O.I of approximately 10. Virus infected cells were collected after observation of apparent CPE. EPC cells were collected at 21 h PI. Collected virus-infected cells were pelleted with low speed centrifuge and fixed with 2.5% glutaraldehyde/4% paraformaldehyde solution overnight at 4^oC. Fixed cells were washed, dehydrated, infiltrated and embedded via standard preparation procedure. EM samples were observed with Hitachi H-7000 transmission electron microscope.

3.3 Results

3.3.1 Expression of GIV genes

RT-PCR was used to study the expression of three GIV genes during the first 12 h PI of the experimental cell lines: EPC, CHSE-214 and RTS11 (Fig 3.1). Two genes, encoding for a tumor necrosis factor receptor (TNFR) domain (030L) and a predicted E3 ubiquitin ligase domain (097L), were known to be expressed early in the GIV replication cycle, and the third one was the major capsid protein gene (MCP), which was known to appear late in replication. Expression of the viral genes was first confirmed in the GK cell line, with amplified transcripts being sequenced and verified by nucleotide BLAST from NCBI to be from GIV (data not shown). In GK, the early genes (030L and 097L) were expressed at the earliest point examined, 1 h, and expression of the late gene (MCP) was first seen at 6 h (Fig 3.1). The same pattern of expression was seen in EPC. For CHSE-214, expression began later, at 6 h for the early genes and at 12 h for the late gene. For RTS11, only the early genes were expressed and this began at 6 h. Therefore, as manifested by the expression of the two early genes, GIV can at least begin its infection cycle in EPC, CHSE-214, and RTS11.

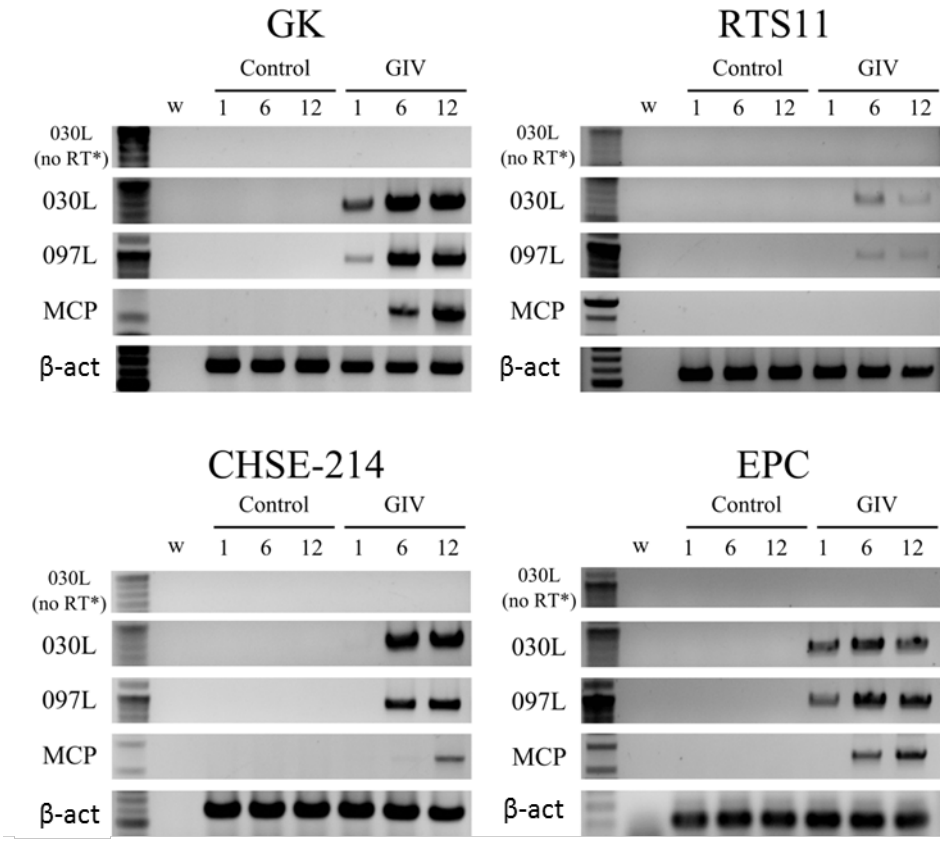

Fig. 3.1 Expression of GIV transcripts in RTS11, CHSE-214 and EPC

Two transcripts, a tumor necrosis factor receptor (TNFR) domain (030L), and a predicted E3 ubiquitin ligase domain (097L) expressing at an early stage in GIV replication, and one transcript, the major capsid protein gene (MCP), expressing at a late stage of replication were probed in GIV infected GK, RTS11, CHSE-214 and EPC cells. Samples were taken at time points of 1, 6, and 12 h. (w) Lane is the water control. Transcripts, 030L and 097L, appeared very early (1 h PI) in GK and EPC, and later (6 h PI) in RTS11 and CHSE-214. MCP was not detected at all in RTS11 but was seen abundantly in GK and EPC at the 6th hour and in CHSE-214 at the 12th hour. β-act was used as a positive control for reverse transcription-PCR. As a control for potential DNA contamination from the RNA preparation, one set of samples from the same RNA preparation was subjected to the reverse transcription process without reverse transcriptase (RT) in the reaction. When these samples were later probed with 030L, there were no PCR products amplified, signifying no viral DNA contamination.

3.3.2 Induction of apoptosis by GIV

By 12 h PI, GIV caused signs of cell death in cultures of the four cell lines (Fig. 3.2b, d, f and h), while mock-infected cultures appeared healthy (Fig. 3.2a, c, e, and g). Infected cultures in RTS11, CHSE-214 and EPC developed debris and floating cells with a characteristic feature: the cells were phase dark with a bright center (see arrows in Fig. 3.2d, f, and h). For RTS11, video microscopy was used to document a culture from the point of GIV introduction to the appearance of the floating dead cells (see supplementary material). The mode of cell death, whether necrosis or apoptosis, was investigated further using DNA laddering and Annexin V-PE staining assays.

Genomic DNA was isolated from infected and control cultures at approximately 12 h PI, separated by electrophoresis, and visualized with ethidium bromide. After the addition of GIV, DNA laddering was clearly evident in cultures of the three non-host cell lines, but not in GK cells (Fig. 3.3). Aliquots of GIV were subjected to heating and UV irradiation treatment, and examined for their capacity to induce apoptosis. To confirm significant viral reduction, GK cells were infected with either heat-, UV-, or non-treated GIV as described in materials and methods. Heat treatment completely inactivated GIV as there was no titre produced by GK cells 3 days PI (data not shown). UV irradiation greatly reduced GIV titre to a very small amount of <10 $TCID_{50}$/mL after 3 days PI; although not complete inactivation, titre was 6 logs below that of the GK cells infected with non-treated GIV ($10^{6.33}$ $TCID_{50}$/mL). For all cell lines, exposure to heat-inactivated GIV failed to cause DNA laddering (Fig 3.3). With UV-treated GIV (GIV_{uv}), the responses depended on the cell line. For GK and CHSE-214, GIV_{uv} did not cause DNA laddering; by contrast, for EPC and RTS11, GIV_{uv} caused DNA laddering (Fig. 3.3). These results suggest that GIV induces apoptosis in RTS11, CHSE-214, and EPC.

Confirmation of apoptosis in RTS11, CHSE-214 and EPC was sought by examining cultures for phosphatidylserine translocation. An early hallmark of apoptosis, the translocation of PS from the inner to the outer leaflet of the plasma membrane, was assayed in the three non-host cell lines at approximately 12 h PI. This was done through the use of Annexin V conjugated with a fluorophore to identify phosphatidylserine on the external surface. Cells were stained with Annexin V-PE and 7-AAD and the number of cells with externalized phosphatidylserine determined by flow cytometry [21]. A representative output is shown in Fig. 3.4 for EPC. In the

figure, data was collected as a percentage of the entire cell population and cells that are stained with only Annexin V-PE (quadrant B4) are in early apoptosis.

The differences in the amount of apoptosis for the three non-host cell lines at various treatment conditions were determined in two independent experiments and are shown in Table 3.2. For all cell lines, heat-inactivated GIV treatment resulted in either lower or similar level of apoptosis as the controls. For cell lines treated with normal GIV, the apoptosis level was much higher relative to untreated controls: RTS11 apoptosis level at 13.0% and 15.5% compared to 3.1% and 5.7% in controls ($p<0.05$), CHSE-214 at 16.8% and 18.7% compared to 4.0% and 5.3% in controls ($p<0.01$), and EPC at 8.3% and 9.3% compared to 2.7% and 2.0% in controls ($p<0.01$). Cell lines treated with GIV_{uv} produced varied results that depended on the cell line. Compared to the previously stated apoptosis levels of control cells, RTS11 treated with GIV_{uv} had a slight increase in apoptosis in two independent experiments at 9.3% and 7.5%, a result supported by DNA fragmentation in Fig. 3.3. CHSE-214 cells challenged with GIV_{uv} had apoptosis levels at 3.5% and 5.9%, similar to control cells, and this result was supported by the lack of DNA fragmentation in Fig. 3.3. When compared to control cells, EPC apoptosis level significantly increased at 13.2% and 14.9% ($p<0.001$) when treated with GIV_{uv}, and interestingly, was higher than in EPC treated with normal GIV (8.3% and 9.3%, $p<0.05$). The Annexin V-PE staining results paralleled those of the DNA laddering assay, confirming the findings that GIV induces apoptosis in all the non-host cell lines. However, the differential results of each cell line to GIV_{uv} suggest that GIV induces apoptosis by distinctive routes in different cell lines.

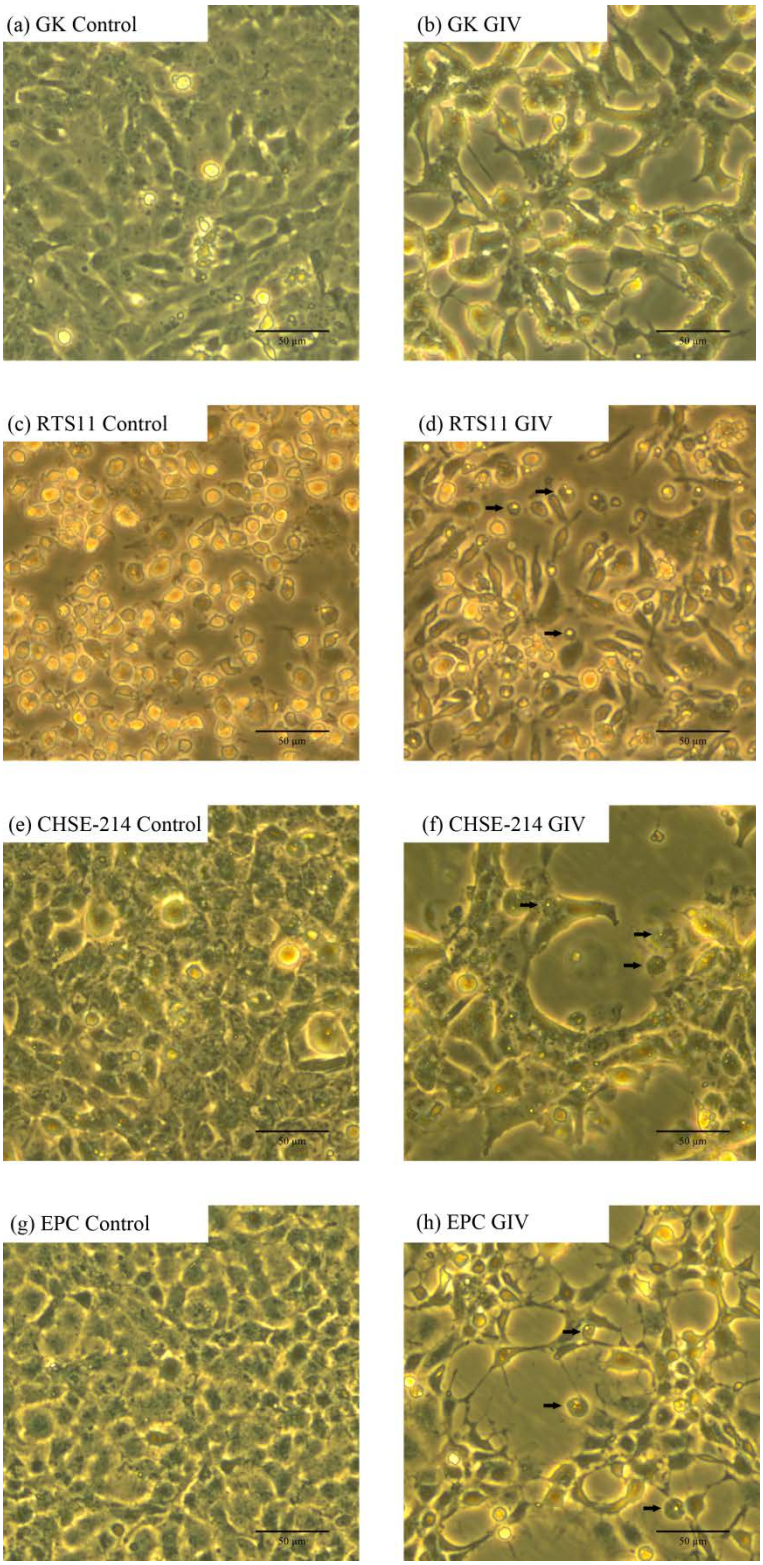

Fig. 3.2 GIV induced cell death in GK, RTS11, CHSE-214 and EPC

Cell monolayers from these cell lines were either mock infected or infected with GIV, and incubated at 20 °C overnight. Mock-infected cells, GK (a), RTS11 (c), CHSE-214 (e) and EPC (g) appeared normal for each cell line. RTS11 is a cell line that has both suspended and attached cells. In the GIV infected cells, there are considerable cell death in all cell lines; however, RTS11 (d), CHSE-214 (f) and EPC (h) showed significant cellular debris that appears phase dark with bright centers (arrows) while infected GK cells (b) showed little to none. The scale bar represents 50 μm.

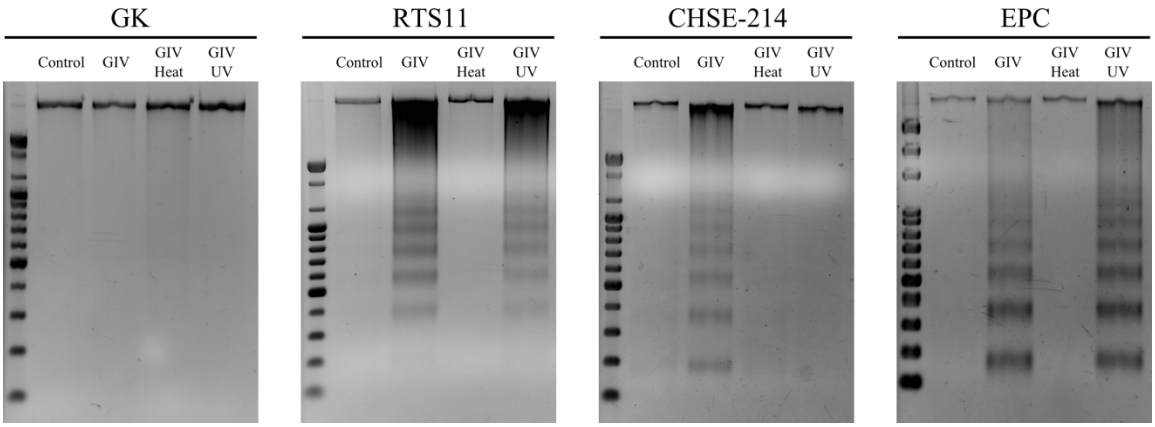

Fig. 3.3 Induction of DNA fragmentation in RTS11, CHSE-214 and EPC

GK, RTS11, CHSE-214 and EPC cells were either mock-infected, or infected with infectious GIV, heat- or UV-treated GIV. DNA samples of infected cells were collected and ran on a 1.8% agarose gel. Normal GIV, heat-inactivated GIV and UV treated GIV was unable to induce DNA fragmentation in GK cells; however, normal GIV induced DNA laddering in RTS11, CHSE-214 and EPC. Heat-inactivated GIV did not cause DNA fragmentation in any of the cell lines. Exposure of RTS11 and EPC with UV-treated GIV did result in DNA laddering, while exposure of GK and CHSE-214 did not. The first lane of each gel shows the DNA size makers.

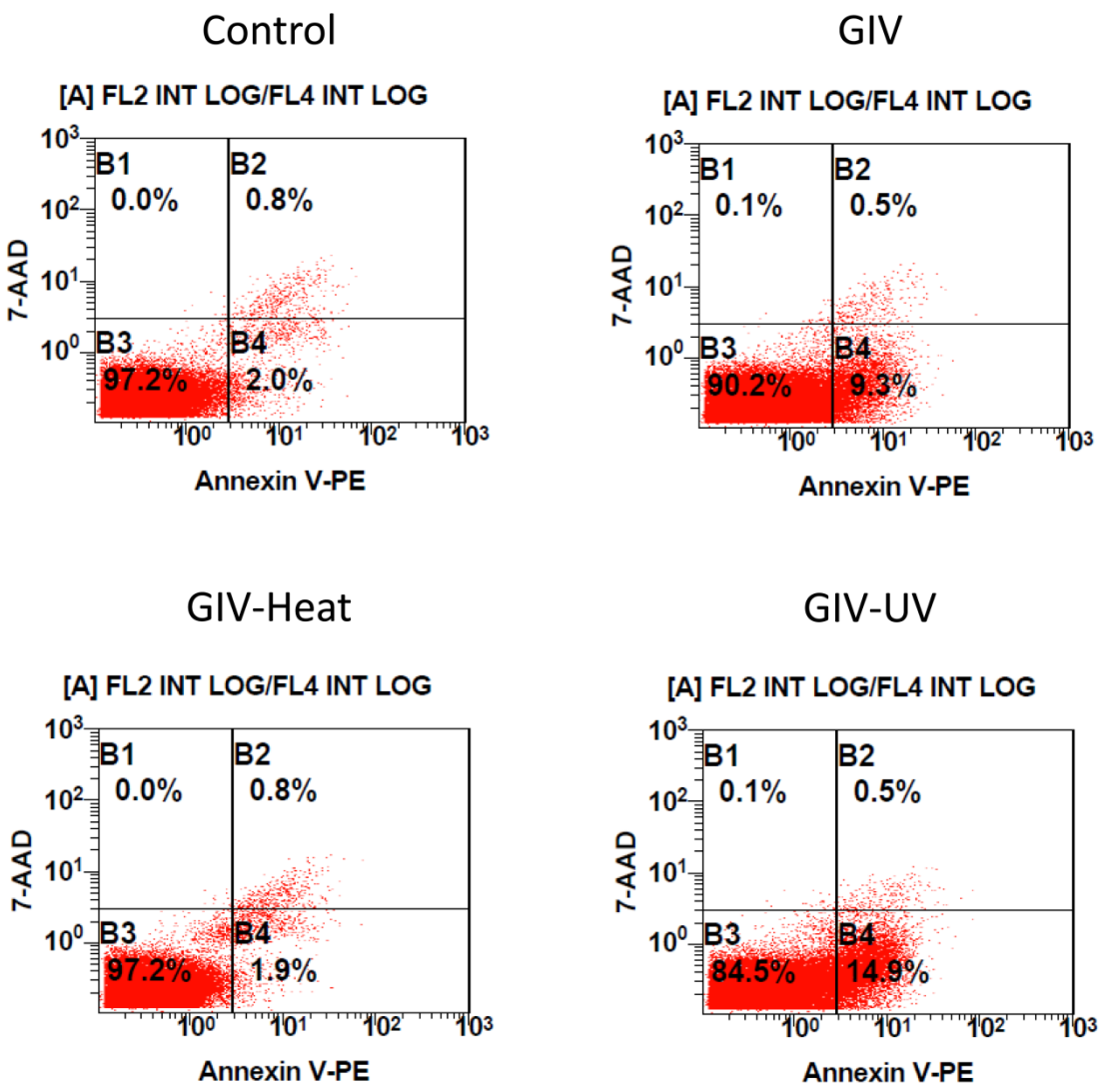

Fig. 3.4 Representative example of Annexin V-PE staining of EPC cells under various treatments

For each graph, the y-axis represents percentage of cells stained by 7-AAD, which stains for necrotic cells, and the x-axis represents percentage of cells stained by Annexin V-PE, which stains for early apoptotic cells. Cells that are only stained with 7-AAD (quadrant B1 on the graphs) are necrotic. Cells stained with only Annexin V-PE (B4) are in early apoptosis. Cells stained with both 7-AAD and Annexin V-PE (B2) cannot be differentiated between necrosis or late apoptosis. Cells that do not stain with either 7-AAD or Annexin V-PE are viable. For this example, the percent of apoptotic cells in EPC challenged with normal GIV and UV-treated GIV is 9.3% and 14.9% respectively, in contrast to 2.0% for control medium and 1.9% for heat-inactivated GIV.

Table 3.2 Annexin V-PE staining of RTS11, CHSE-214 and EPC cells either mock infected, or infected with normal GIV, heat- or UV-treated GIV

		% of cells in early apoptosis			
Cell Line	**Replicates**	**Control**	**GIV**	**GIV-Heat**	**GIV-UV**
RTS11	1	3.1	13.0	4.5	9.3
	2	5.7	15.5	4.6	7.5
CHSE-214	1	4.0	16.8	3.5	3.5
	2	5.3	18.7	5.1	5.9
EPC	1	2.7	8.3	1.7	13.2
	2	2.0	9.3	1.9	14.9

Four separate treatments were prepared for each cell line. In the control treatment, cells were mock infected with 2% FBS/L15. For the various virus treatments, cells were either infected with normal GIV, heat-inactivated GIV (2 hrs at 60 ^{o}C) or UV-treated GIV (150 mJ). Each cell line was exposed to the treatments for approximately 12 h before the cells were stained with Annexin-V PE and measured using flow cytometry.

3.3.3 Replication of GIV

To determine whether GIV can replicate in RTS11, CHSE-214 and EPC, cultures of these cell lines were exposed to GIV for 1 h, rinsed, returned to growth medium, and monitored for titre for up to 7 days afterwards. The titres of GK-infected cultures were also monitored as a positive control for GIV replication. The average titre in GK-infected cultures increased from a day zero value of $10^{4.37}$ $TCID_{50}$/mL to $10^{8.70}$ $TCID_{50}$/mL by day 3, greater than 4 logs increase. At day 3, there was complete destruction of GK monolayer; therefore, day 7 titre was not collected. EPC cultures also supported GIV replication but were less productive. Over a 7-day period, the titre of GIV in EPC increased from an average of $10^{5.28}$ $TCID_{50}$/mL (day zero) to $10^{6.20}$ $TCID_{50}$/mL (day 3), to $10^{7.28}$ $TCID_{50}$/mL (day 7) (Fig. 3.5); there was an average of 2 logs increase in titre by day 7 relative to day zero. By contrast, GIV titre in RTS11 and CHSE-214 did not increase from day zero to day 7 (Fig. 3.5). Therefore, EPC cultures produced GIV but RTS11 and CHSE-214 cultures did not, indicating that apoptosis could be induced in RTS11 and CHSE-214 without the completion of viral replication. The diverse responses of the fish cell lines to GIV are summarized in Table 3.3.

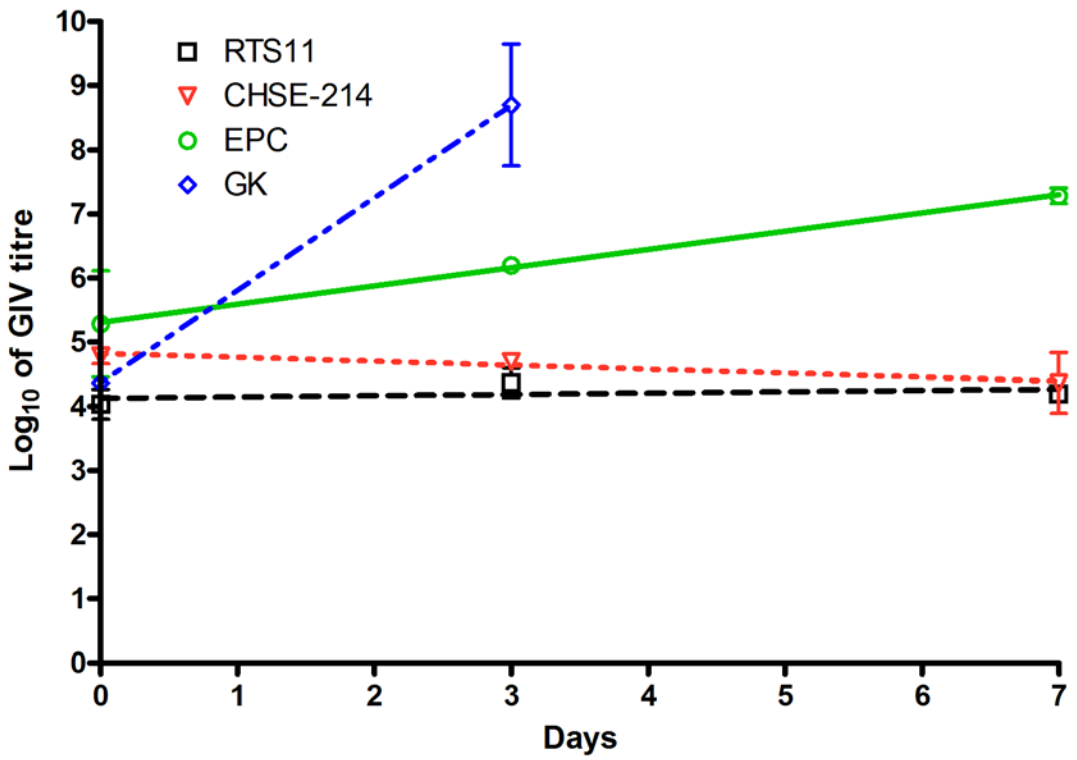

Fig. 3.5 Replication of GIV in GK, RTS11, CHSE-214 and EPC

GK, RTS11, CHSE-214 and EPC were infected with GIV and monitored over 7 days for viral replication. To determine viral titre, $TCID_{50}$ was performed on supernatant samples taken at time points of day zero, 3, and 7 for RTS11, CHSE-214, and EPC. For GK cells, samples were taken up to day 3. The $TCID_{50}$/mL value of each time point was log transformed. The increase in GIV titre is determined to be significant for GK ($p < 0.05$) and EPC ($p < 0.01$) by linear regression analysis; the slope of the line is significantly non-zero.

Table 3.3 Summary comparison of each cell line response to GIV infection

Cell line	Apoptosis			Viral gene expression		Virion production
	GIV	GIV-Heat	GIV-UV	Early	Late	
GK	-	-	-	+	+	+
RTS11	+	-	+	+	-	-
CHSE-214	+	-	-	+	+	-
EPC	+	-	+	+	+	+

This table summarizes the results presented and offers a global view of the key differences in the interaction of each cell line with GIV. (+) represent a definitive “yes” and (-) a “no” of the cells response in each category. The degree of each response is described in details in the results section.

3.3.4 Transmission Electron Microscopy (TEM) of GIV-infected EPC cultures

When EPC cultures were examined by TEM at 21 h PI with GIV, virions were clearly observed within the cytoplasm of some cells (Fig. 3.6). Cells were seen with hexagonal shaped structures of approximately 150 nm. Some of these had an inner electron dense region surrounded by a less dense region and a hexagonal shell. These are interpreted as complete virions. Others lacked the inner electron dense region (indicated by arrows) and had an incomplete shell. These are interpreted as incomplete virions. Both types of virions were clustered in paracyrstalline arrays, which likely represent viral maturation sites. Overall these results confirm that GIV replicate in EPC.

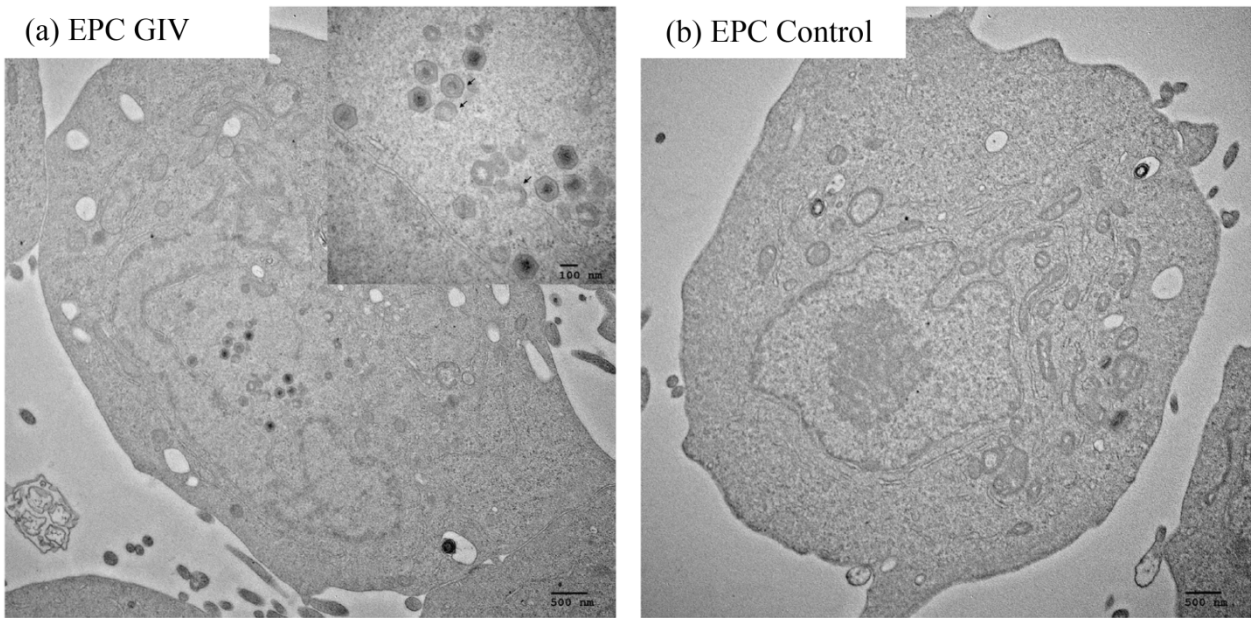

Fig. 3.6 Electron microscopic observation of GIV virions in EPC cells

EPC infected (a) or uninfected (b) with GIV were observed under electron microscope. Virions were only observed in infected EPC. Complete virions showed electron dense nucleocapsid structure in the middle of the icosahedral viral capsids. Incomplete virion (indicated by arrows) showed viral icosahedral capsids that were less dense.

3.4 Discussion

Grouper iridovirus (GIV) has been shown for the first time to express genes and induce apoptosis in three cell lines, RTS11, CHSE-214 and EPC, from three non-host species, respectively rainbow trout (*O. mykiss*), Chinook salmon (*O. tshawytsch*), and fathead minnow (*P. promelas*). The GIV transcripts were for two early genes, tumor necrosis factor receptor (TNFR) and a predicted E3 ubiquitin ligase domain, and one late gene, the major capsid protein (MCP). The pattern of GIV gene expression differed among cell lines. Expression of all three genes occurred earlier in EPC than in CHSE-214, whereas only weak expression of the early genes was seen in RTS11. Among the three non-host cell lines, only EPC produced GIV with a moderate yield over 7 days of infection. By contrast, the host cell line, GK from the yellow grouper *E. awoar*, had a similar time course of GIV gene expression as EPC but produced much more GIV, while did not undergo apoptosis early in the infection cycle. Therefore, at the cellular level the fathead minnow would appear to have the potential to be a host.

Unlike the GK cell line, the non-host cell lines underwent apoptosis, but the circumstances were different for each line, particularly in response to GIV_{UV}. Relative to GIV, GIV_{UV} increased apoptosis in EPC, reduced apoptosis in RTS11, and caused no apoptosis in CHSE-214. To explain these differences, two assumptions are made. The first is that GIV virions or protein(s) alone have the capacity to induce apoptosis, without the need for gene expression. The second is that the cell lines differ in the extent to which they support expression of the GIV genome and how the expressed viral proteins regulate their apoptotic pathways. These two assumptions are used below to explain the responses in EPC and CHSE-214, which contrast with each other, and then RTS11, which might be an intermediate situation.

As GIV_{UV} is expected to support minimal viral gene expression due to very low infectious titre, but yet significantly increased apoptosis in EPC cultures, GIV virions and/or polypeptides appear to be capable of alone inducing apoptosis early on infection of these fathead minnow cells. This interpretation is supported by a previous study with GIV and by reports with at least two very different iridoviruses, FV3, which is a *Ranavirus*, and CIV (Chilo iridescent virus), which is an insect iridovirus. GIV and GIV_{UV} induced apoptosis in the barramundi cell line, BM, whereas heated-treated GIV did not [22]. For FV3, both UV- and heat-treated virus induce apoptosis in fish cell cultures [23]. These results suggest that virions or virion proteins induced apoptosis. This was confirmed more directly with CIV in a spruce budworm cell line. As well

as CIV_{UV}, an extract of CIV capsid protein (CVPE) was able to induce apoptosis [24], possibly by activating an apical caspase, designated *Choristoneura fumiferanai* initiator caspase (*Cf*-caspase-i) [25]. To cause apoptosis, CIV, CIV_{UV}, and CVPE had to be internalized by low-pH-endocytosis [25]. *De novo* viral protein synthesis was not required for the apoptosis initiated by CIV_{UV} and CVPE. With untreated CIV, apoptosis was reduced by early viral gene expression and increased by inhibiting protein synthesis with cycloheximide [25]. These results suggest that CIV codes for anti-apoptotic factors and their expression can reduce apoptosis by this route. Returning to grouper-like iridoviruses, GIV is known to code for anti-apoptotic Bcl [26]. Thus the increase in apoptosis in GIV_{UV} infected EPC cultures is likely due to the suppression of viral anti-apoptotic genes by the UV treatment. By contrast in GIV infected EPC cultures, anti-apoptotic gene expression early upon infection would reduce apoptosis at the beginning and allow some cells to survive and support the production of GIV.

As GIV_{UV} caused no apoptosis in CHSE-214, cell death early during the infection of these cultures with GIV must require the virus to enter the cells, become uncoated and express genes that trigger apoptosis. Triggering could be through specific pro-apoptotic proteins, which might be coded for in the GIV genome [27] or through general viral protein synthesis, which might trigger apoptosis through the unfolded protein response [28]. The anti-apoptotic mechanisms of early GIV infection must be either incapable of overriding these triggers or be poorly expressed in CHSE-214. Why CHSE-214 failed to respond to the virions and polypeptide(s) of GIV_{UV} could be due to lack of specific surface receptors or an appropriate internalization route. In the case of the insect iridovirus, low-pH-endocytosis either of CIV, CIV_{UV}, or CVPE was necessary for apoptosis [25]. Such a pathway might be used preferentially for the internalization of UV treated viruses and be impaired in CHSE-214.

For RTS11, apoptosis might be triggered both by GIV virions and/or polypeptide(s) and by early GIV gene expression. These cells had an intermediate response to GIV_{UV}. Relative to GIV, GIV_{UV} only reduced the level of apoptosis. As some apoptosis was still induced by GIV_{UV}, RTS11 would appear to be dying in response to GIV virions and/or polypeptide(s). However, the UV treatment did not completely inactivate all of the GIV. Therefore a small amount of GIV gene expression might also be contributing to this apoptosis.

Grouper-like *Ranaviruses* appear to modulate the life of host and non-host cells differently, but whether other *Ranaviruses* do so requires further study. In the current study, the host/non

host distinction is suggested by the behavior of GK cells, which produced the virus but did not die by apoptotic death during the early period of viral production. Many *Ranaviruses* have been seen to bud from cells during viral propagation [29], implying that cells can release infectious virus without dying. The first suggestion of a unique death mode for host cells has come with SGIV. SGIV caused apoptosis in the cell line FHM, but not in grouper spleen cells (EAGS) [30]. FHM was derived from fathead minnow, which is not a known SGIV host; EAGS, from a SGIV host, the Hong Kong grouper, *E. akkara*. Although SGIV killed a portion of the cells in EAGS cultures, death was non-apoptotic and instead resembled paraptosis-like death [30]. In contrast to this report, SGIV caused greasy grouper embryonic cells (GEC) to undergo apoptosis [31]. However, in this case a purified intracellular form of the virus was used for infection [31]. The most studied *Ranavirus*, FV3, can be propagated on cell lines from several different fish species, including fathead minnow [32,33] and caused apoptosis in at least one fish cell line, FHM, and in a mammalian cell line, BHK [23]. None of these cell lines would be considered as being from FV3 hosts. In fact, cell lines from anuran hosts appear not to have been used to study FV3.

In the future, the cell lines of this report along with other cell lines should be useful for studying the different routes by which *Ranaviruses* are internalized and the links to gene expression, apoptosis and viral propagation. Understanding these phenomena might explain host range at the cellular level and indicate the potential for host range expansion. Monocyte/macrophage cell lines would be of particular interest as macrophages may contribute to FV3 transmission by harbouring quiescent viruses [34]. In this context the killing of monocyte/macrophage RTS11 by GIV might reflect a cellular barrier to the establishment of this virus in salmonids. Although the barriers to infection likely reside at multiple levels of biological organization and are modulated by many factors, understanding barriers at the cellular level will allow a complete picture of the mechanisms involved in determining host tropisms and over the long-run help in studying the significance of each.

CHAPTER 4

Ranavirus, Frog virus 3 (FV3) infections of rainbow trout cells lead to apoptosis-dependent cell death in macrophages but apoptosis-independent cell death in other cell types*

*This chapter has been submitted to the journal *Fish & Shellfish Immunology*.

4.1 Introduction

The ability of teleost fish to act as asymptomatic carriers for an amphibian *Ranavirus*, Frog virus 3 (FV3), had been recently suggested in infection trials with pike *Esox lucius*, pike-perch *Sander lucioperca* and rainbow trout *Oncorhynchus mykiss* [1,2,3]. However, detailed reports of the tissue and cell type that is susceptible or permissive to FV3 infection in carrier fish is lacking, but is important for understanding the mechanism behind the ability of fish to act as potential reservoir species. FV3 was originally isolated from a tumour of leopard frogs (*Rana pipiens*) [4]; it mainly infects amphibians and had been associated with the decline of the global amphibian population [5]. Incidentally, a strain of a *Ranavirus* had been simultaneously isolated from both amphibian and fish in the wild [6], indicating that one strain of *Ranavirus* is capable of infecting multiple species. Indeed, further evidences suggest that *Ranaviruses* have undergone multiple host shift events in their history and are considered to be multi-host pathogens; however, the ancestral *Ranavirus* is believed to be from fish [7, 8].

Ranavirus is a genus of viral species that belongs to the *Iridoviridae* family. Viruses in the *Iridoviridae* family are large (120 – 180 nm), icosahedral in shape, and contain a double-stranded DNA genome [9]. The *Iridovirdae* family contains five genera: *Iridovirus*, *Chloriridovirus*, *Lymphocystivirus*, *Ranavirus*, and *Megalocytivirus*, whose natural hosts include invertebrates and lower vertebrates; *Ranaviruses* mainly infect fish, amphibians and reptiles [9]. The virions exist as both naked and enveloped forms. The naked form consists of an outer protein coat encapsulating the viral genome, allowing the virion to enter the host cell through direct fusion with the cell membrane, while the enveloped form contains a lipid membrane surrounding the protein capsid, allowing virion entry by receptor mediated endocytosis [9] or the recently suggested caveolae-mediated endocytosis [10]. These two different virion forms and subsequent routes of entry may be one mechanism allowing *Ranavirus* to have a broad host range.

In most studies on cultures of insect, fish or mammalian cell lines, viruses in the *Iridoviridae* family have induced apoptosis, but there are exceptions, and the results hint at host and non-host cells responding differently. For example, cell lines from spruce budworm *C. fumiferana* and boll weevil *Anthonomous grandis* underwent apoptosis when infected with Chilo iridescent virus (CIV) [11]. Fathead minnow cells (FHM) underwent apoptosis when infected with FV3, *Rana grylio* virus (RGV), and Singapore grouper iridovirus (SGIV) [12,13,14]. In addition, FV3 also induced apoptosis in baby hamster kidney (BHK) [12], and baby green monkey kidney (BGMK)

cells [15]; grouper iridovirus (GIV) in Chinook salmon embryonic cells (CHSE-214), fathead minnow (EPC), rainbow trout monocyte/macrophage (RTS11) [16], and barramundi *Lates calcarifer* (Bloch) muscle [17] and swimbladder cells [18]; and SGIV in medaka haploid ES cells [19]. In all of the above cases, the cells that underwent apoptosis are all non-natural host cells. Interestingly, GIV and SGIV induced apoptosis-independent cell death in host grouper kidney (GK) [16] and grouper spleen (EAGS) cells [14]. The permissiveness of cells to support *Ranavirus* infection may depend on resistance to viral induction of early apoptosis and this can be one of possibly many potential mechanisms for determining the reservoir status of a fish species.

In this report the response of different rainbow trout cells in culture to FV3 has been evaluated in an attempt to understand the response of rainbow trout to FV3. Five adherent cell lines were used: these were the epithelial-like RTgill-W1 from the gill [20], RTL-W1 from the liver [21], RTgut-GC from the intestine [22], the fibroblast-like RTG-2 from the gonad [23] and RTHDF (hypodermal connective tissue) from skin [24]. The sixth cell line was RTS11, which is from the spleen, grows loosely over the culture surface, and has monocyte/macrophage properties [25]. Only RTG-2 had been investigated previously with FV3 and was found to weakly support virus replication but cell death was not studied [26]. FV3 caused death in all of the rainbow trout cell lines but only the monocyte/macrophage cell line RTS11 appeared to die by apoptosis. In primary rainbow trout leukocyte cultures FV3 also killed macrophages but spared lymphocyte-like cells. When two neoplastic human cell lines, U937 [27], and PEER [28] were exposed to FV3, cell death was induced in the macrophage-like U937 cells but not in the lymphocyte-like PEER cells. FV3 production was supported by RTG-2 and to a lesser extent RTL-W1, but not by any of the other rainbow trout cell lines.

4.2 Materials and Methods

4.2.1 FV3 propagation and quantification

Frog virus 3, FV3 was obtained from Dr C. Brunetti at Trent University, Peterborough, Ontario [29]. The virus was propagated and titred on EPC, an epithelial-like cell line from fathead minnow [29,30]. Propagation was done on EPC cells in 75 cm^2 flasks (BioLite, Thermo Scientific) at 26^oC in L15 medium with 2% fetal bovine serum (FBS) (PAA Laboratories, VWR International). Seven days post infection (PI), the entire content of the flasks was collected and centrifuged at 4000 xg for 5 min to pellet cellular debris. The supernatant was collected, syringe filtered through a 0.2 µm filter (Pall Corporation, Thermo Scientific), separated into aliquots and stored at -80^oC. Viral titre was determined using the Karber method of tissue culture infectious dose ($TCID_{50}$/mL) [31].

4.2.2 Cell lines

Immune and non-immune cell lines from fish and humans were used. The immune cell lines were RTS11 from rainbow trout and U937 and PEER from humans. 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 [25]. U937 was established from a histocytic lymphoma, grows in suspension, has many properties of macrophages, and is often described as macrophage-like [27,32,33]. PEER originated from a case of T-cell leukemia, grows in suspension, has properties of T cells and is described as a T-cell line [28,34]. 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 [21], RTgut-GC from the gastrointestinal tract [22], and RTgill-W1 from the gill [20]. The two fibroblast-like cell lines were RTG-2 from gonad [23] and RTHDF from skin [24].

The foundation of the growth media was one of two basal media, either Leibovitz's L15 or RPMI-1640 (Hyclone, Thermo Scientific). These were supplemented with FBS and 1 % penicillin-streptomycin (PS) (Hyclone, Thermo Scientific). RTgill-W1, RTL-W1, RTgut-GC, RTG-2 and U937 were grown in L-15 with 10% FBS. For RTHDF and RTS11, the L15 was supplemented with 15% FBS. All of the above cell lines were grown in an atmosphere of air but

at room temperature for the fish cell lines and at 37 °C for U937. PEER also was grown at 37 °C but in RPMI-1640 with 10% heat-inactivated FBS and an atmosphere of 5% CO_2. Cell lines were subcultivated at a ratio of 1:2 once every one to two weeks for fish cells or once every 3 days for human cells.

4.2.3 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 and those that grew in suspension. Cells that grew in suspension were RTS11, U937 and PEER. Adherent cells were detached with trypsin-versene (Lonza, Thermo Scientific) 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 1x with 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 xg for 5 min and washed 1x 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 containing fish cell lines were incubated at 20^{o}C and those containing mammalian cell lines at 37^{o}C for either observation of cytopathic effects (CPE), or further downstream experiments such as resazurin cell viability assay, apoptosis assays, or RT-PCR.

4.2.4 Preparation of rainbow trout immune cell suspensions

Rainbow trout with average weight of approximately 250 g (Lyndon Hatchery) were anesthetized in approximately 1 mL/L of 2-phenoxyethanol (Sigma-Aldrich). Peripheral blood was taken from the caudal vein with a 22G needle coated with heparin (Sigma-Aldrich). The blood was layered over Histopaque-1077 (Sigma-Aldrich) and centrifuged at 400 xg for 30 min. The cells at the serum/Histopague-1077 interface were collected and transferred to a second Histopaque-1077 layer for another 30 min centrifuge at 400 xg. The cells at the interface were collected again, washed 2x with D-PBS, 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 xg for 30 min. The cells at the interface were collected, washed 2x with D-PBS, and resuspended in L15 with 10% FBS for subsequent initiation of primary cell cultures and experiments.

4.2.5 Preparation and infection of rainbow trout primary immune cell culture with FV3

From the head kidney and peripheral blood leukocyte 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 x 10^6 cells/mL. The plates were incubated overnight at 14^{o}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 were 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 were 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 cultures from peripheral blood and head kidney leukocytes were labeled as PBL-A and HKL-A, respectively. Non-adherent cultures from peripheral blood and head kidney leukocytes were respectively labeled as PBL-F and HKL-F. Adherent and non-adherent cultures were infected with FV3 as described for the cell lines, incubated at 20^{o}C, and evaluated for cell viability and gene expression as outlined below.

4.2.6 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 4.2.3 and 4.2.5, 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 [35]. 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 [35,36]. A commercial preparation of resazurin called alamarBlue was used (Life Technologies Inc, Burlington, ON). 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 relative fluorescent units (RFU) and percent metabolic impairment was determined by dividing the RFU of FV3 exposed wells with RFU of control wells and multiplying by 100.

4.2.7 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 4.2.3. 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 xg for 5 min and total genomic DNA was extracted using the E.Z.N.A Tissue DNA Kit (Omega bio-tek, VWR International). 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). Quantification of apoptotic cells was conducted using a Beckman Coulter flow cytometer (Cytomics FC 500 MPL), software version MXP-v2.2.

4.2.8 Evaluating the ability of cell lines and primary cultures to support FV3 gene expression

Cells and total primary culture head kidney leukocytes were infected as described in Section 4.2.3 and Section 4.2.5, 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 xg 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) or Promega ImProm-II Reverse Transcription System (Promega, Thermo Scientific). 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'-CTCCTTAATGTCACGCACGATTTC-3') [37]; and major capsid protein (MCP) gene (forward primer 5'-GACTTGGCCACTTATGAC-3' and reverse primer 5'-GTCTCTGGAGAAGAAGAA-3') [38]. The cycling conditions were as follow: initial denaturation at 95^{o}C for 3 min; 40 amplification cycles of 94^{o}C for 1 min, primer annealing at 55^{o}C for 45 s, and elongation for 45 s; and a final extension at 72^{o}C for 7 min. PCR products were electrophoresed on a 1% agarose gel and subsequently imaged. RT-PCR was performed on 2 independent experiments for each cell line.

4.2.9 Evaluating the ability of rainbow trout cell lines to support FV3 production

Cell lines were infected with FV3 as described in Section 4.2.3, and incubated for 2 h before the old medium was removed. The cells were then washed 3x 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 xg 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^{o}C for subsequent titration. After collection, 100 µL of new medium was added back into the wells and the cells were incubated at 20 ^{o}C for up to 10 days. 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) [31].

4.3 Results

4.3.1 FV3 production in rainbow trout cells

Only two rainbow trout cell lines, the fibroblastic RTG-2 and epithelial RTL-W1, appeared to support production of FV3 (Fig. 4.1). EPC, the positive control cell line used to propagate the virus, produced the highest average level of titre, approximately $10^{9.70}$ $TCID_{50}$/mL by day 10 PI which is significantly higher than the titre left over following the initial washing of the virus on day zero ($10^{3.87}$ $TCID_{50}$/mL; Fig. 4.1). RTG-2 produced the second highest average FV3 titre of approximately $10^{7.45}$ $TCID_{50}$/mL by day 10 PI compared to day zero titre of $10^{4.53}$ $TCID_{50}$/mL (Fig. 4.1). The average titre of FV3 in RTL-W1 was third highest and reached $10^{5.70}$ $TCID_{50}$/mL by day 10 PI, which is more than 1 log higher than the day zero titre ($10^{4.12}$ $TCID_{50}$/mL; Fig. 4.1). However, FV3 titres in the rest of the adherent rainbow trout cell lines (RTgill-W1, RTgut-GC, and RTHDF) were all less than 1 log from their day zero titre by day 10 PI, indicating that these cell lines do not support a significant amount of FV3 replication. RTS11 cultures failed to support an increase in FV3 titre over 10 days of infection (Fig. 4.1).

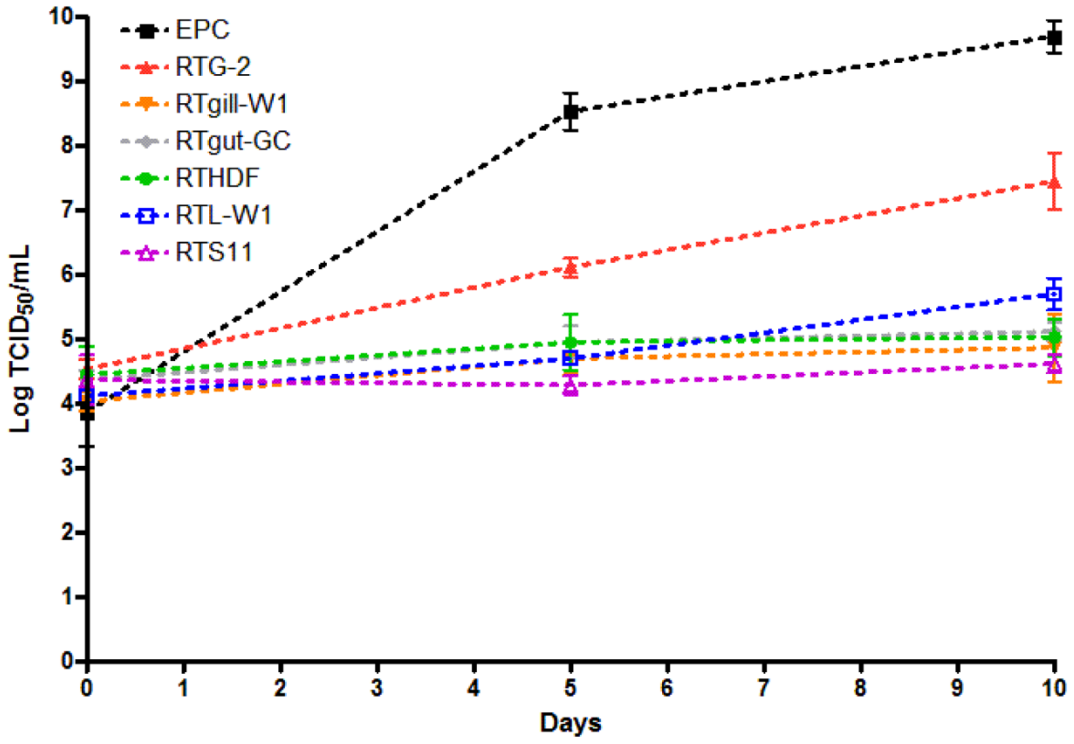

Fig. 4.1 Titre of FV3 produced in rainbow trout cell lines and EPC

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 one 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 ± SD.

4.3.2 Major capsid protein (MCP) gene expression in rainbow trout cells

To determine if FV3 genes can be expressed in rainbow trout cultures of immune origin, transcripts of the late major capsid protein (MCP) gene were examined by RT-PCR in control and infected RTS11 and total primary HKL. RTS11 and the rainbow trout primary HKL expressed MCP transcripts at 20 ^{o}C (Fig. 4.2). Even though the RNA preparations were treated with DNase, there were some remaining background viral genomic DNA in the no reverse transcriptase (RT) control preparations that showed a random pattern of appearance; however, the intensity of the bands in the no RT control was much lower than samples that contained RT.

For adherent rainbow trout cell lines at 20°C, both fibroblast (RTG-2 and RTHDF) and epithelial (RTL-W1, RTgut-GC, and RTgill-W1) cell lines expressed FV3 MCP transcripts (Fig. 4.2).

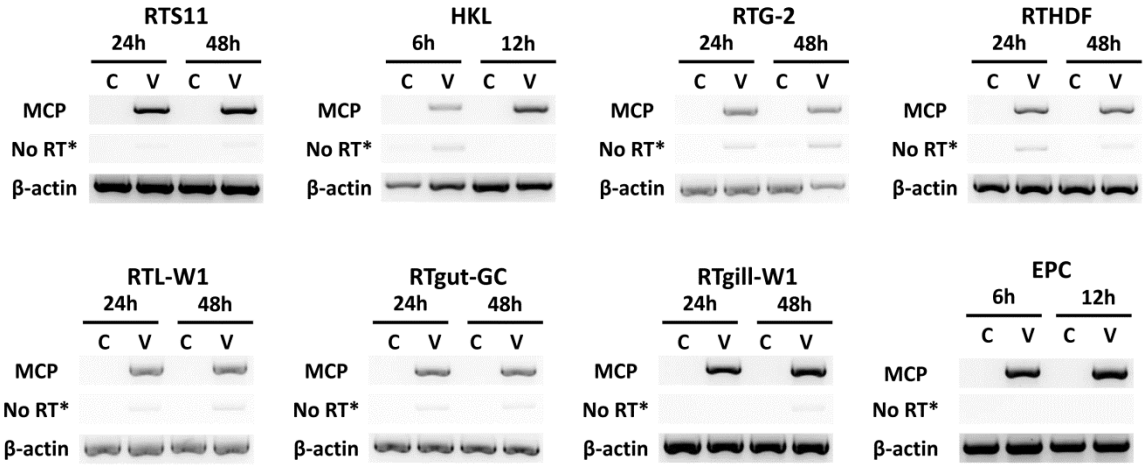

Fig. 4.2 Expression of FV3 transcripts in cell lines and primary cultures

One transcript, the major capsid protein (MCP) expressed at the late stage of the FV3 replication cycle was probed in the rainbow trout cell lines, total primary head kidney leukocytes (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. C represents lanes with control cells and V represents lanes with FV3 infected cells.

4.3.3 CPE of FV3 on rainbow trout cells

Upon infection with FV3, all cultures of the adherent cell lines developed damage to the cell monolayers when compared to mock infected control cells. RTG-2 (Fig. 4.3a) and RTHDF (Fig. 4.3b) monolayers showed significant damage by 24 h; RTL-W1 (Fig. 4.3c), RTgut-GC (Fig. 4.3d) and EPC (Fig. 4.3e) by 48 h; and RTgill-W1 (Fig. 4.3f) by 72 h post infections (PI).

FV3 was detrimental to cultures of the macrophage-like cells that adhered from suspensions of peripheral blood leukocyte (PBL) and the head kidney leukocyte (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. 4.4a, b). By contrast, cultures of non-adherent cells with FV3 continued to look healthy, with little cellular debris and similar to control cultures not exposed to FV3. 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 showed no change in the ability to reduce alamarBlue (Fig. 4.4c), and those from the HKL showed only a slight drop, with capacity at 80 to 90% of the control level (Fig. 4.4c). By contrast, the reduction of alamarBlue by adherent macrophage-like cultures from PBL and HKL dropped significantly, with respectively 40 to 50% and 10 to 20% of control level at 24 h PI with FV3 (Fig. 4.4c).

FV3 also appeared to kill the macrophage-like cell line, RTS11. Over 24 h, cultures with FV3 were observed by phase contrast microscopy and seen to accumulate cellular debris and phase dark cells (Fig. 4.4d). Also the ability to reduce the indicator dye, alamarBlue, dropped dramatically. Relative to control cultures, FV3 infected cultures reduced alamarBlue at 50 to 60 % of the level of control cultures at 24 h PI and at 72 h PI this had dropped to at 20 to 30% (Fig. 4.4c). Therefore FV3 appeared to kill adherent macrophage-like cells from primary culture and the monocyte/macrophage cell line, RTS11.

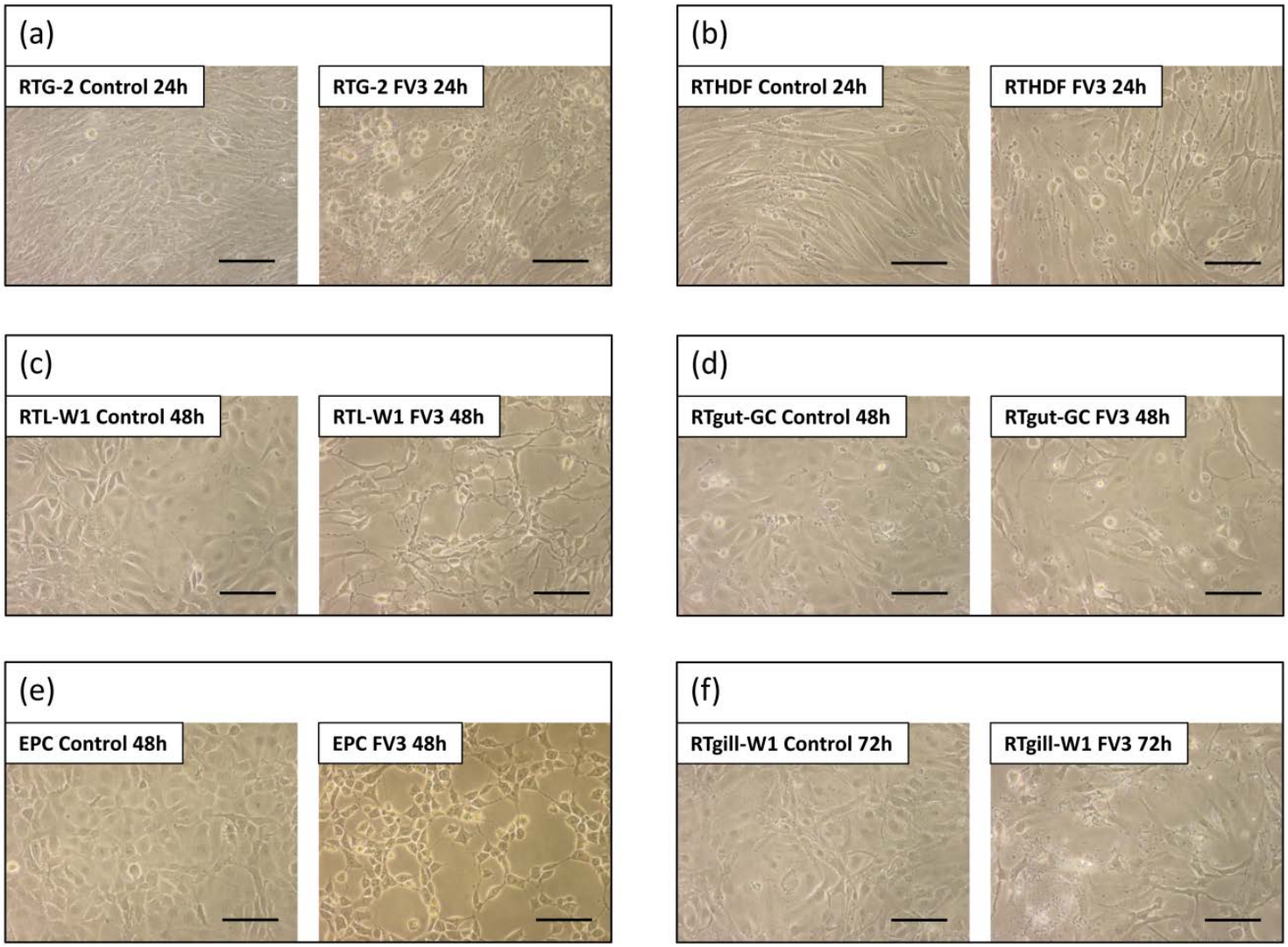

Fig. 4.3 Observations of cytopathic effect (CPE) in adherent fish cell lines infected with FV3

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.

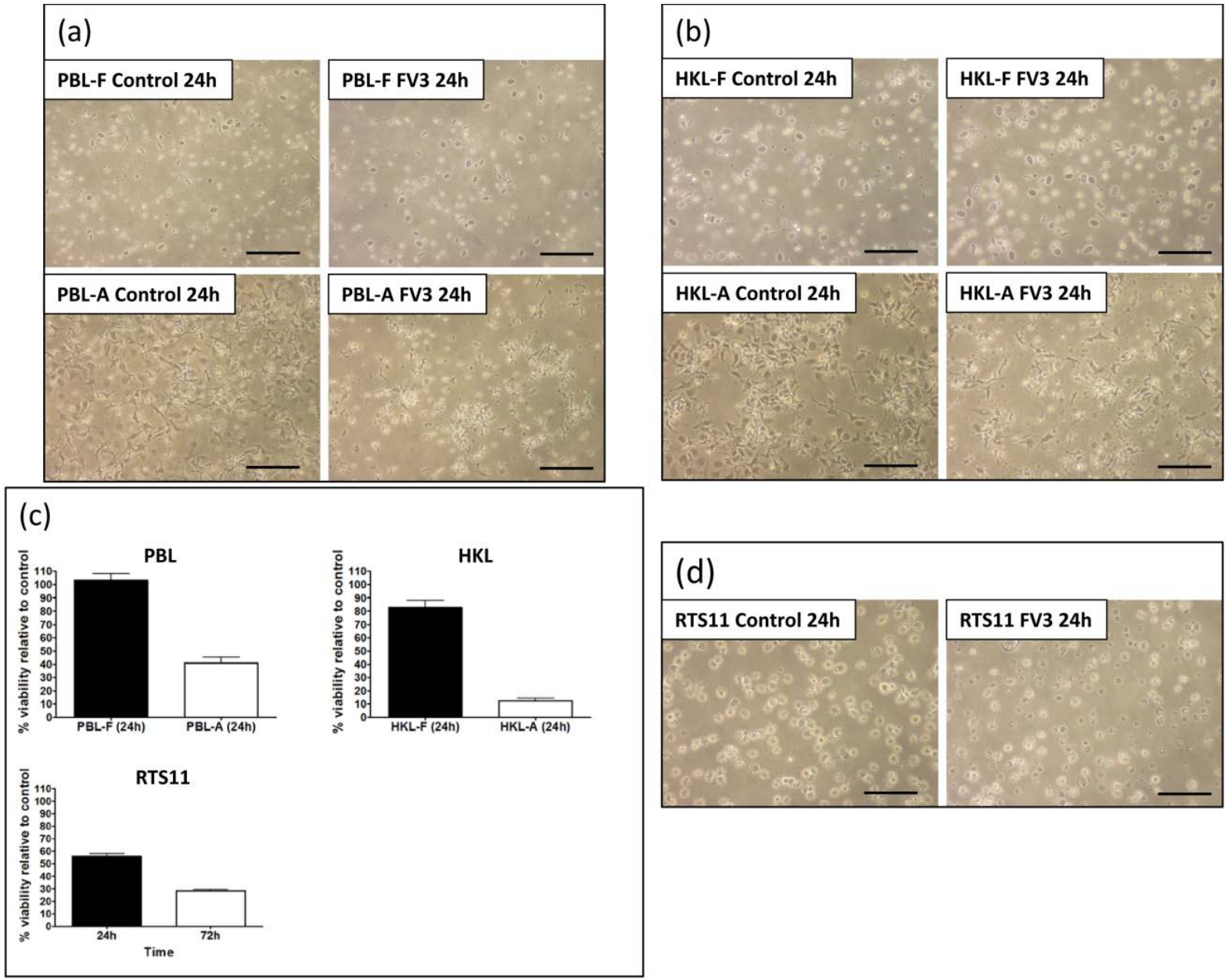
(a)
PBL-F Control 24h
PBL-F FV3 24h
PBL-A Control 24h
PBL-A FV3 24h
(b)
HKL-F Control 24h
HKL-F FV3 24h
HKL-A Control 24h
HKL-A FV3 24h
(c)
PBL
% viability relative to control
PBL-F (24h)
PBL-A (24h)
HKL
HKL-F (24h)
HKL-A (24h)
RTS11
24h
72h
Time
(d)
RTS11 Control 24h
RTS11 FV3 24h

Fig. 4.4 Observations of cytopathic effects (CPE) and metabolic activity of rainbow trout primary leukocyte cultures infected with FV3

Peripheral blood leukocytes (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 leukocytes (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 ^{o}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 ± SD. The scale bars represent 100 µm.

4.3.4 Induction of apoptotic hallmarks in rainbow trout cells by FV3

For cultures in which cells died, the mode of cell death caused by FV3 was investigated, beginning with RTS11 cultures at 20 °C. 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. 4.5a); the 48 h PI time point was not shown for RTS11 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 on RTS11 infected with FV3 and mock infected control cultures. RTS11 cultures infected with FV3 showed an average of approximately 6.40% of annexin V positive cells at 24 h PI while base level in control ranges from an average of 1.14-3.27% (Table 4.1). A representative output from the flow cytometer for RTS11 is shown in Fig. 4.5b; quadrant Q3 indicates the percent of cells in early apoptosis. Therefore, FV3 caused the death of RTS11 by apoptosis.

FV3 failed to induce DNA laddering in cultures of the rainbow adherent cell lines, whether fibroblastic or epithelial (Fig. 4.5a). As DNA laddering was not seen, these cultures were not examined for Annexin V-PE staining. The adherent cell lines, whether fibroblastic or epithelial, appeared to die by an apoptosis-independent mechanism.

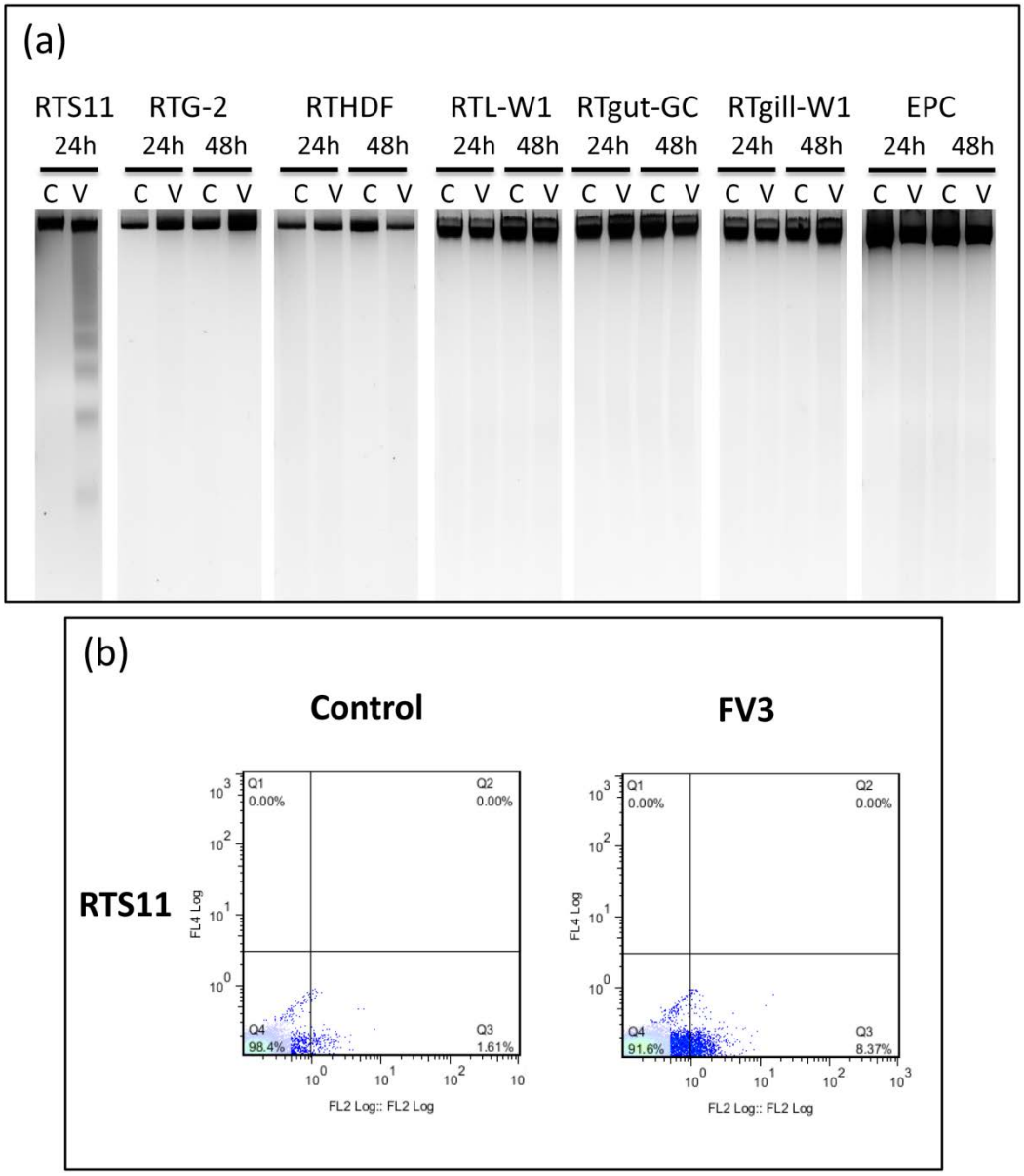

Fig. 4.5 Detection of apoptosis in cell lines

(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) Representative flow cytometer output of RTS11 cells infected with either FV3 or control medium. Q3 (bottom right quadrant of each graph) shows the percentage of cells in early apoptosis. The FL4 Log y-axis showed cells stained with 7-AAD while the FL2 Log x-axis showed cells stained with Annexin V-PE.

4.3.5 Interactions of human immune cell lines with FV3

MCP transcripts were detected in PEER cultures infected with FV3 at 37°C (Fig. 4.6a). They were not detected in U937 at intensities beyond that of the no RT control (Fig. 4.6a). Therefore, U937 were unique among the cell lines of this study in not expressing FV3 MCP transcripts.

FV3 caused CPE, as indicated by the impaired ability to reduce alamarBlue and phase contrast observations, in both U937 and PEER cultures at 37 °C but in a subtly different manner. The capacity to reduce alamarBlue was decreased to 70 to 80% of control value in U937 cultures and 60 to 70% in PEER cultures at 72 h PI (Fig. 4.6b). Small subcellular debris accumulated over 24 h in U937 cultures infected with FV3, but not to the same extent in the control; however, infected PEER cultures looked very similar to control (Fig. 4.6c). Therefore FV3 appeared to impair metabolism but not kill the T cells (PEER) whereas a portion of the macrophages (U937) were killed.

FV3 also induced apoptosis in U937 cultures at 37°C. DNA laddering was observed at 24 and 48 h PI (Fig.4.6d). Annexin V-PE staining also suggested that U937 died by apoptosis. U937 cultures infected with FV3 showed an average of 17.97-18.87% of annexin V positive cells at 72 h PI while the level in control cells ranges from an average of 12.43-13.47% (Table 4.1).

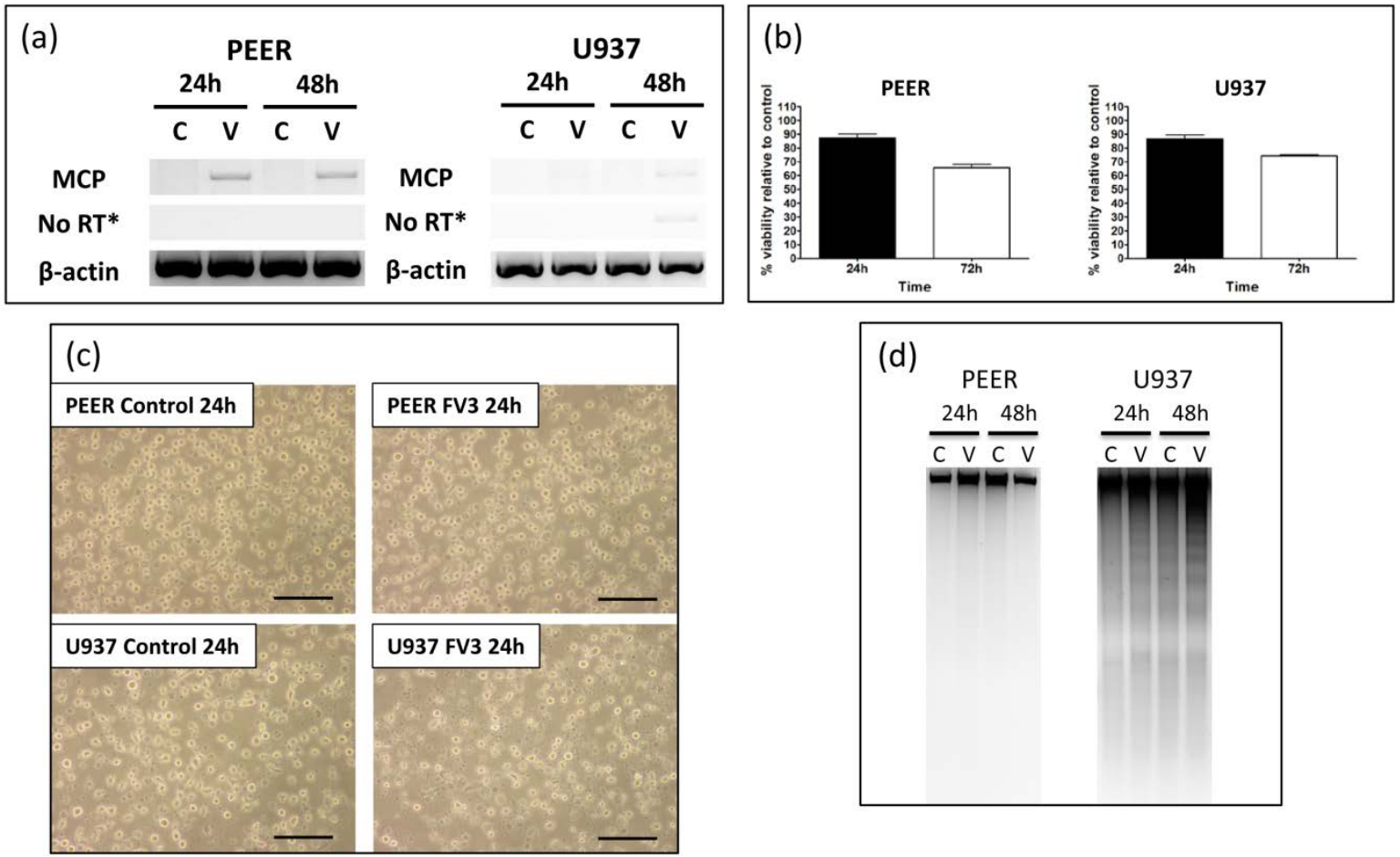

Fig. 4.6 Infections of human cell lines with FV3

(a) Expression of FV3 major capsid protein (MCP) transcripts in PEER and U937 control or FV3 infected cells at 24 h and 48 h post infection (PI). (b) Metabolic activity, as representative of cell viability, of PEER and U937 cells infected with FV3 at 24 h and 72 h PI relative to their control counterpart. Data points represent n = 3 ± SD. (c) Phase contrast observations of PEER and U937 control or FV3 infected cells at 24 h PI. (d) DNA laddering of PEER and U937 control or FV3 infected cells at 24 h and 48 h PI.

Table 4.1 Annexin V-PE staining of RTS11 and U937 control and FV3 infected cell cultures.

Average % of cells in early apoptosis (± SD)			
RTS11	**Control**	**FV3**	**Statistical significance**
Trial 1 (triplicates)	3.27 (± 0.63)	6.42 (± 0.91)	$p < 0.01$
Trial 2 (triplicates)	1.14 (± 0.46)	6.40 (± 1.79)	$p < 0.01$
U937	**Control**	**FV3**	**Statistical significance**
Trial 1 (triplicates)	13.47 (± 0.40)	18.87 (± 0.25)	$p < 0.0001$
Trial 2 (triplicates)	12.43 (± 0.21)	17.97 (± 0.15)	$p < 0.0001$

RTS11 and U937 were infected with FV3 for 24 h and 72 h, respectively, before being stained with Annexin V-PE and 7-AAD, and quantified with a flow cytometer. Mock infected control cells were done in parallel. Cells that stained for Annexin V-PE but not 7-AAD were considered to be in early apoptosis. Two independent trials were performed for each cell line with each trial done in triplicates. Statistical analyses for all trials were performed using the Student's t test.

4.4 Discussion

Rainbow trout cell lines from various tissues and primary leukocyte cultures from the head kidney and peripheral blood were used to examine the potential of rainbow trout to produce Frog Virus 3 (FV3), support FV3 gene expression, and to experience FV3-induced pathogenic changes. Only two cell lines produced FV3, and production was poor. Yet all cell lines and primary cultures supported major capsid protein (MCP) gene expression, and except for lymphocyte-like primary cultures, the cell lines and primary macrophage cultures underwent cytopathic effects (CPE) that included cell death. The apparent difference between the response of rainbow trout lymphocytes and macrophages to FV3 led to human lymphocyte (PEER) and macrophage (U937) cell lines being studied as well. In both rainbow trout and human cells, FV3 killed macrophages but not lymphocytes, and the mode of cell death was consistent with apoptosis.

4.4.1 FV3 production in rainbow trout cell lines

The rainbow trout cell lines supported either slight or no FV3 production. FV3 replicated only in RTG-2, a gonadal fibroblast cell line [23], and RTL-W1, a liver epithelial cell line [21]. The FV3 titre produced on either cell line was several orders of magnitude lower than on EPC, which is a fathead minnow cell line commonly used to propagate the virus. Another study also found RTG-2 to be one of the least effective of five fish cell lines at supporting *Ranavirus* propagation [26]. Here RTG-2 was better than RTL-W1. Four other rainbow trout cell lines failed to support a one log increase in FV3 titre. These were two epithelial cell lines, RTgill-W1 from the gill [20] and RTgut-GC from the gut [22], one fibroblast cell line, RTHDF from skin [24], and a monocyte/macrophage cell line RTS11 from the spleen [25]. The cell line results would suggest that rainbow trout cells have only a slight capacity to produce FV3. One possible explanation for low FV3 titre in rainbow trout cells is inefficient codon usage by FV3 during the viral replication cycle as was experimentally observed for FV3 infection in mammalian cells [29]; if the codons carried by the FV3 genome is not the preferred or optimal codon used by rainbow trout cells then the inefficiency in translation may be the cause of low FV3 titre. *In vivo* this property would be expected to reduce the spread of the virus and allow easier clearance of the virus by the fish immune system. In fact FV3 appears only weakly infectious to rainbow

trout. When 50 rainbow trout fry were inoculated with FV3, the virus could be isolated 7 days later from only 1 fish and no pathological changes were observed [3].

The failure of most rainbow trout cell lines to support FV3 replication is surprising in light of reports describing FV3 replication in epithelial and fibroblast cell lines from a range of vertebrates. Fibroblast cell lines from bluegill fry (BF-2) and baby hamster kidney (BHK) supported FV3 replication [39], as did primary chick embryo fibroblasts [40]. Similarly, epithelial cell lines such as FHM cells from fathead minnow [40], XTC-2 from frog [41], CHO from Chinese hamster [42], BGMK from buffalo green monkey [29], and HeLa cells from human have all been shown to be permissive for FV3 [43]. However, temperature is a factor in the replication of FV3 in mammalian cells. The permissive temperature for FV3 replication in mammalian cells ranged from 12^{o}C to 32^{o}C [40]; beyond that, virions failed to assemble [44,45].

4.4.2 Major capsid protein (MCP) gene expression in rainbow trout cells

FV3 was able to enter rainbow trout cells because cultures of all rainbow trout cell lines and of primary leukocytes expressed the MCP gene post infection. This gene is expressed late in FV3 replication, suggesting that the transcriptional steps of the viral life cycle can take place in rainbow trout cells. Other steps would appear to be impaired.

4.4.3 CPE of FV3 in rainbow trout cells

Despite being produced only slightly or not at all in rainbow trout adherent cell lines, FV3 caused CPE. The CPE was the rounding of cells and their concurrent death. In cultures of adherent cell lines from a range of vertebrates FV3 caused a similar type of CPE [29,40-43]. In these cases CPE was associated with FV3 production. By contrast, for most of the rainbow trout cell lines FV3 failed to be produced, despite the CPE. Possibly FV3 caused CPE in these cell lines by acting like a toxin, inducing cell death by the act of binding to cells or entry into cells rather than from the events of FV3 gene expression and completing its replication cycle. Previously an extract of FV3 particles was found to be detrimental to an epithelial cell line, inhibiting RNA synthesis [46]. Additionally, an insect iridovirus has been reported to kill cells by a virion protein binding to a cell membrane receptor [47,48]. The RTG-2 and RTL-W1 cell lines might be less sensitive to the toxin-like effect of FV3, allowing some FV3 production.

FV3 caused CPE in cultures of the monocyte/macrophage cell line RTS11 and of primary macrophages but not of lymphocytes. For macrophages, CPE was seen as the appearance of cellular debris in cultures and as a decline in metabolism as measured with alamarBlue, an oxidation-reduction dye. Living cells reduce alamarBlue and a decline in their capacity to do so indicates an impairment of cellular metabolism and/or cell death [35,36]. In contrast to macrophages, lymphocytes showed no change in metabolism upon exposure to FV3 for 24 h. Thus among rainbow trout leukocytes FV3 appears to act selectively on macrophages.

4.4.4 Induction of apoptotic hallmarks in rainbow trout cells by FV3

For the monocyte/macrophage cell line, RTS11, FV3 induced apoptosis, but for the fibroblast and epithelial cell lines, the CPE was likely mediated by a different cell death pathway(s). RTS11 had been previously shown to undergo apoptosis when infected with another *Ranavirus*, grouper iridovirus (GIV) [16] and together, these results suggest apoptosis as a common response of rainbow trout macrophages to *Ranavirus* infections; however, further infection studies of RTS11 or other rainbow trout macrophages with additional *Ranaviruses* is necessary to confirm this hypothesis. FV3 has been reported to cause DNA laddering in cultures of the fathead minnow epithelial cell line, FHM [12]. Therefore the lack of laddering with the rainbow trout epithelial and fibroblast cell lines is surprising and might reflect differences in cellular infections between the species. Recently, the types of cell death have increased, with several possibilities beyond either apoptosis or necrosis [49]. Whether FV3 kills the rainbow trout fibroblast and epithelial cell lines by necrosis or some other mode needs to be investigated in the future.

4.4.5 Interactions of human immune cell lines with FV3

To investigate whether in other species FV3 acts differently on macrophages than on other leukocytes, the human T (PEER) and monocytic (U937) cell lines were investigated at 37°C and were found to act very differently. In PEER cultures, metabolism declined 24 h PI but the cells were viable and expressed MCP transcripts. By contrast, in U937 cultures at 24 h PI, metabolism declined, cellular debris appeared, and MCP transcripts were absent. The lack of MCP transcripts in U937 had several possible causes. The virus might not have been able to enter these cells by the normal route of infection. Alternatively FV3 entered U937 but these

cells either failed to support transcription or transcript accumulation. In other systems, FV3 transcripts were shown to be unstable, with a short half-life, and degrade quickly if not continually transcribed [50]. The U937 death was by apoptosis because DNA ladders and phosphatidylserine externalization were seen after infection.

The killing of U937 by FV3 appears to result from the virus acting like a toxin rather than being a consequence of virus transcription, replication, and/or egress. This is suggested by the lack of MCP transcript accumulation in U937 and by previous reports of mammalian cells failing to support FV3 replication at 37°C [40]. Interestingly, an extract of FV3 particles was found to be detrimental to HeLa cells, inhibiting RNA synthesis [46]. Finally other *Iridoviruses* have been reported to kill cells by a virion protein binding to a cell membrane receptor [47,48]. In addition to induction of apoptosis by viral proteins, the lipid components of the FV3 envelope are potential inducers of apoptosis in U937 cells. These cells were previously shown to be very sensitive to induction of apoptosis by lipid compounds; alteration of cellular membrane fluidity was hypothesized as the mechanism for apoptosis induction by lipids [55]. The U937/FV3 interaction is another example of macrophage/*Ranavirus* interactions leading to apoptosis but this might not be true for macrophages of all vertebrates. RTS11 underwent apoptosis in response to FV3 in this study and to GIV in a previous study [16]. Peritoneal leukocytes collected from FV3 infected frog *Xenopus leavis*, showed the presence of apoptotic cells, approximately 8% of the population [51]. On the other hand, *X. laevis* macrophages infected with FV3 have been shown to survive for 12 days, which may allow them to act as cellular carriers for FV3 in the host [52].

Interestingly, *in vivo* studies with rodents at 37°C suggest a unique interaction between FV3 and liver macrophages, Kupffer cells. When FV3 was administered to rats through the penis dorsal vein, FV3 caused fatal hepatitis within 24 h [53]. The Kupffer cells were found to have internalized FV3 and then lysed by an unknown mechanism, although the ultrastructure of the Kupffer cells was described as necrotic. An *in vitro* examination of Kupffer cell/FV3 interactions found that they were complex [54]. By 2 h, the dominant interaction was FV3 internalization into phagosomes, primarily by phagocotysis but pinocytosis might have made a small contribution. From the phagosomes some viral particles shed their contents into the cytoplasm. A very small portion of the FV3 appeared to bind to the plasma membrane and liberate their contents into the cytoplasm through stalk-like structures. Whether these

interactions occur with U937 but not with PEER and whether they contribute to U937 apoptosis are questions that would be interesting to answer in the future.

4.4.6 Conclusions

In conclusion, rainbow trout cell lines from various tissues did not support FV3 replication except weakly in the case of RTG-2 and RTL-W1. This overall result would suggest that rainbow trout would be unlikely to contribute to the spread of the virus in the aquatic environment by producing and shedding the virus. However, rainbow trout might still contribute to spread by being asymptomatic carriers of the virus at a low level. Finally, the fact that FV3 entered all rainbow trout cell types tested and caused CPE in most of them suggests that exposure to high FV3 concentrations, such as by consumption of FV3 infected frogs, might be detrimental to rainbow trout health.

CHAPTER 5

Corexit 9500 inactivates two enveloped viruses of aquatic animals but enhances the infectivity of a non-enveloped fish virus*

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5.1 Introduction

Recently chemical dispersants have been introduced into aquatic environments in large quantities because of their use in the cleanup of oil spills. Of the estimated 38 major accidental oil spills since 1970 [1], approximately 20 have been from oil tankers [2]. These spills are often treated with dispersants such as Corexit 9500. The exact composition of Corexit is proprietary but in general terms, the ingredients include anionic surfactants, nonionic surfactants, and organic solvents [3-5]. The dispersants function by breaking down large oil pools into small oil droplets, allowing them to sink into the water column. The small oil particles are diluted by waves into very minute concentrations [6]. The Deepwater Horizon oil spill in the Gulf of Mexico resulted in historically, the largest volume of dispersant ever used in one incident; approximately 7.57 million liters (mainly Corexit 9500) was used to disperse the oil, exposing the immediate and surrounding biological environment to the dispersant [7].

With the increase in the volumes of dispersant used, more attention is being given to the impact of dispersants on the health of humans and the environment. For human health, the research has ranged from clinical assessments [8] to studies on cell lines [9]. For the environment, the possible influence of dispersants has been investigated on a range of metazoans, including corals, crustaceans, mollusks, fish, and birds [7,10,11]. Even effects on the microbial world are being examined. For example, Corexit might interfere with the capacity of bacteria from beaches to remediate spills [12]. Perhaps the one component of aquatic environments that has never been studied for interactions with Corexit is viruses.

Viruses are the most abundant biological entities in the aquatic environment and tremendously important in ecological processes as well as being the causative agents of many diseases [13,14]. All the types of viruses in water constitute the virosphere. The most common viruses target bacteria. These bacteriophages or phages influence the microbial loop and ultimately global biogeochemical cycles [15]. Although greatly outnumbered by phages, viruses that infect algae and aquatic animals are also in the sea and freshwater [16,17]. Whether from prokaryotes or eukaryotes, viruses have common structural themes. All viruses have capsids that enclose either RNA or DNA genomes. Viruses that additionally have a lipid membrane (envelope) are referred to as enveloped viruses, whereas those that have just the capsid are naked or non-enveloped viruses. Of these two groups, Corexit might be expected to impact enveloped viruses because surfactants have been reported to inactivate some enveloped viruses [18].

In this report, the effect of Corexit 9500 has been studied on four aquatic viruses that infect fish but differ in structure. These are the rhabdovirus, viral hemorrhagic septicemia virus (VHSV), the birnavirus, infectious pancreatic necrosis virus (IPNV), the reovirus, chum salmon reovirus (CSV), and the iridovirus, frog virus 3 (FV3) [17]. VHSV is an enveloped virus with an RNA genome. IPVN and CSV are naked viruses with RNA genomes. FV3 is a DNA virus but the viral particles (virions) can be either naked or enveloped. The viruses were exposed to Corexit 9500 for 24 h and titred on fish cell lines. Corexit inactivated VHSV and FV3, depending on the dispersant concentration, but had no effect on IPNV. Surprisingly Corexit activated CSV. These results suggest that Corexit has the potential to alter the aquatic virosphere.

5.2 Materials and Methods

5.2.1 Cell lines propagation

Two cell lines used in this study were EPC, recently reclassified as from fathead minnow [19], and CHSE-214. Both cell lines were grown in 75 cm^2 flasks (BD Biosciences, Fisher Scientific) using Leibovitz's L15 medium (Hyclone, Fisher Scientific, Mississauga, ON) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, VWR International, Mississauga, ON) and 1 % penicillin-streptomycin (PS) (Hyclone, Fisher Scientific, Mississauga, ON).

5.2.2 Virus propagation and quantification

Four viruses used in this study were viral hemorrhagic septicemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), chum salmon reovirus (CSV), and frog virus 3 (FV3). VHSV and IPNV were obtained from Dr. J. Lumsden and Dr. R. Stevenson at University of Guelph, Guelph, Ontario, respectively; FV3 from Dr. C. Brunetti at Trent University, Peterborough, Ontario; and CSV from the American Type Culture Collection (ATCC). VHSV and FV3 were propagated and titred on the EPC cell line. IPNV and CSV were propagated and titred on the CHSE-214 cell line. Propagation was done on confluent monolayers of either EPC or CHSE-214 in 75 cm^2 flasks in L15 with 2% FBS. IPNV and CSV were propagated at 18^oC, VHSV at 14^oC and FV3 at 26^oC. At approximately seven days post infection (pi), the supernatant and cells were collected, and centrifuged at 4000 xg for 5 min to pellet cellular debris. The supernatant was collected and syringe filtered through a 0.2 µm filter (Pall Corporation, Fisher Scientific, Mississauga, ON) and stored at -80^oC. The Karber method of tissue culture infectious dose ($TCID_{50}$/mL) was used to determine viral titre [20].

5.2.3 Determining the effect of Corexit 9500 on cell lines

The cytotoxic effect of Corexit 9500 on EPC and CHSE-214 was determined using two methods: the fluorometric resazurin assay and the colourmetric crystal violet assay. Resazurin is an oxidation-reduction indicator dye that can be reduced by living cells in the process of cellular metabolism. The reduction of resazurin produces a fluorescent product that can be measured as

relative fluorescent unit (RFU). Healthy cells produce higher RFU than dead or dying cells [21]. Crystal violet is a DNA staining dye that can be used to visualize the adherence of cells to multi-well cell culture plates. For cells that have adherent growth characteristics, loss of adherence to the growth surface can be an indicator of cell death [21]. In both assays, the cells were seeded into 96-well plate at approximately 40,000 cells per well and allowed to adhere overnight. Corexit 9500 was serially diluted 10 fold in L15 with 2% FBS to 10^{-6} and added to columns of cells in the 96-well plate. For the crystal violet assay, the cells were incubated with Corexit 9500 dilutions for 24 h at 14 ^{o}C before the wells were washed 1x with Dulbecco's phosphate buffered saline (DPBS) (Lonza, VWR International, Mississauga, ON). Cells were fixed with cold 100% methanol for 10 min. The methanol was removed and the cells were incubated in the crystal violet stain (0.5% crystal violent in 25% methanol) for 10 to 15 min. After the removal of the stain, the wells were washed many times in deionized water to remove excess stain before the plates were dried and imaged using a desktop personal computer scanner. For the resazurin assay, the alamarBlue commercial preparation of resazurin was used (Life Technologies Inc, Burlington, ON). Cells in the 96-well plates were incubated with the serial dilutions of Corexit 9500 for 4 d at 14 ^{o}C before the wells were washed 1x with DPBS. Cells were incubated with 5% alamarBlue solution for 1 h before being measured in a CytoFluor fluorescent plate reader (PerSeptive Biosystems). To determine the percent viability of Corexit 9500 exposed cells, the RFU of exposed wells was divided with RFU of control wells without Corexit and multiplying by 100.

5.2.4 Corexit 9500 preparation and static suspension exposure with viruses

Corexit 9500 (Nalco Energy Services, Sugar Land, TX) was serially diluted (v/v) to the following working concentrations in DPBS: 1:5, 1:50, 1:500, 1:5,000 and 1:50,000. For virus exposure, 100 µL of stock virus was mixed with 100 µL of diluted Corexit (1:5, 1:50, 1:500, 1:5,000 and 1:50,000) to make final Corexit 9500 concentrations of 1:10 or 10%, 1:100 or 1%, 1:1,000 or 0.1%, 1:10,000 or 0.01% and 1:100,000 or 0.001%. In control experiments, 100 µL of stock virus was mixed with 100 µL of D-PBS. VHSV mixtures were incubated for specific time points at 14 ^{o}C, CSV and IPNV at 18 ^{o}C, and FV3 at 20 ^{o}C. At specified time points 100 µL of each mixture sample was collected and virus titre was determined. Viral titre reduction or increase was determined by dividing the titre of virus exposed to Corexit 9500 with the titre of

control virus exposed to only DPBS. The resulting value is log transformed and plotted using the Graphpad Prism 4.0 software. To measure pH, viruses and Corexit were mixed at each of the above concentrations in a total volume of 1,000 µL, and the pH was measured with a Toledo pH meter (Mettler Toledo, Mississauga, ON).

5.2.5 Measurement of titre produced by enhanced CSV (eCSV)

One hundred µL of stock CSV was mixed with 100 µL of diluted Corexit (1:5) to make a final Corexit concentration of 1:10 or 10% and incubated for 24 h. After incubation, 100 µL of the CSV/Corexit mixture was removed and serially diluted with 900 µL of DPBS at each dilution from 10^{-1} to 10^{-4}. The 10^{-4} dilution of CSV/Corexit mixture was added to confluent monolayers of CHSE-214 for 2 h in 12-well plate. After 2 h, the mixture solution was removed and the cells were washed 3x with DPBS. After removal of wash solution, 1 mL of L15 with 2% FBS was added to each well. Immediately upon addition, 100 µL was collected from each well for quantification of the residual amount of CSV that remained after washing (day 0). One hundred µL of fresh L15 with 2% FBS was added to each well, to bring the volume back to 1 mL, before the plate was incubated at 18^{o}C for 7 days. At day 7, 100 µL was collected from each well for quantification of CSV titre.

5.3 Results

5.3.1 Determining Corexit 9500 cytotoxicity and detection limit for $TCID_{50}$ assay

One common issue that arises during the study of the effects of chemical disinfectants on viruses is the simultaneous effects of those disinfectants on cell cultures used to report the presence of viruses. Therefore, it is important to define the lower limit of detection imposed by the toxicity of chemical disinfectants on cell cultures [42]. As Corexit 9500 has been shown to be cytotoxic to mammalian [9] and fish [22] cells in culture, the effect of the oil dispersant on the viability of the two fish cell lines, EPC and CHSE-214, that were used to monitor the viruses after their exposure to Corexit 9500 was examined. Corexit 9500 at starting concentration of 10% (v/v) was serially diluted in a mock $TCID_{50}$ assay from 10^{-1} to 10^{-6}; the 10^{-1} $TCID_{50}$ dilution corresponded to a Corexit 9500 concentration of 1%, 10^{-2} of 0.1%, 10^{-3} of 0.01%, 10^{-4} of 0.001%, 10^{-5} of 0.0001%, and 10^{-6} of 0.00001%. Exposure of cells for up to 4 days to Corexit 9500 at $TCID_{50}$ dilutions of 10^{-3} (0.01% Corexit 9500 concentration) to 10^{-6} (0.00001%) were not cytotoxic to either cell line as determined by the alamarBlue and crystal violet assay (Fig. 5.1). For fish cell cultures receiving $TCID_{50}$ dilutions of 10^{-3} to 10^{-6} or higher, any destruction of cell monolayers would be due to the CPE of the viruses and the viral titre could be determined. However, exposure of cells to $TCID_{50}$ dilutions of 10^{-1} (1% Corexit 9500 concentration) and 10^{-2} (0.1%) resulted in cytotoxicity (Fig. 5.1). Therefore, the lowest limit of virus titre detection in the $TCID_{50}$ assay with 10% initial Corexit 9500 concentration was 1.58×10^3 $TCID_{50}$/mL and with 1% initial Corexit 9500 concentration was 1.58×10^2 $TCID_{50}$/mL.

5.3.2 Determining the pH of virus/Corexit 9500 mixtures

To ensure that any potential effects of Corexit 9500 on the viruses were not due to extreme acidic or basic conditions, the pH of all virus/Corexit mixtures was measured and determined to fall in the range of 7.32 – 8.16. Therefore, any effects of Corexit 9500 on the examined viruses were not due to extremely acidic or basic pH.

5.3.3 Effect of Corexit 9500 on a virus with a single protein capsid, IPNV

The titre of IPNV was not greatly altered by Corexit 9500 at any of the concentrations examined, even after 24 h of exposure (Fig. 5.2).

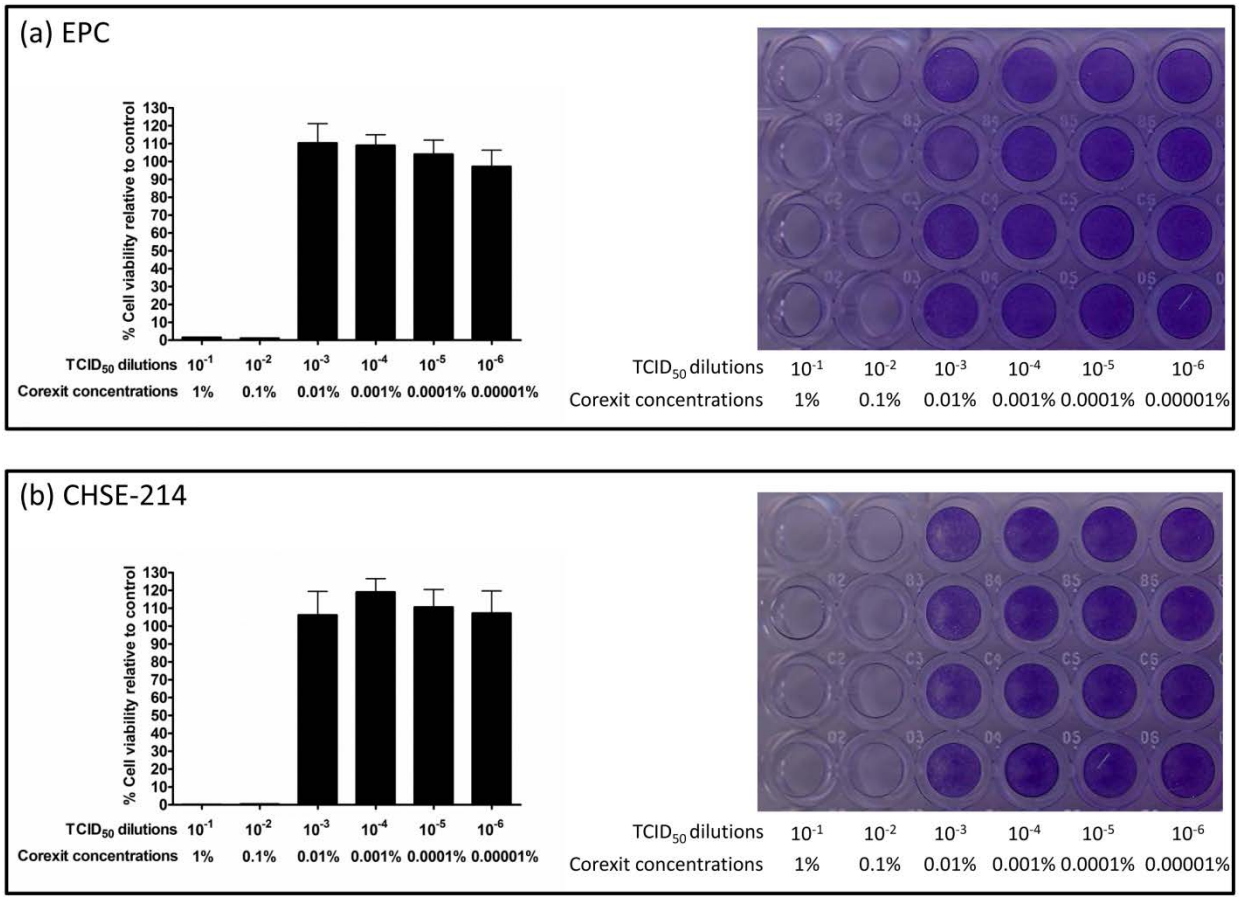


Fig. 5.1 Cytotoxic effect of Corexit 9500 on EPC and CHSE-214

EPC (a) and CHSE-214 (b) were exposed to 10 fold serial $TCID_{50}$ dilutions, up to 10^{-6}, of Corexit 9500 (10% initial concentration) in L15 with 2% FBS before cell viability was determined using the alamarBlue resazurin assay or the crystal violet assay. In the alamarBlue assay (left panel of each figure) the cells were incubated with Corexit 9500 dilutions for 4 d. The points on the graph represent means ± SD, n = 4. In the crystal violet assay (right panel of each figure) the cells were incubated with Corexit 9500 for 24 h.

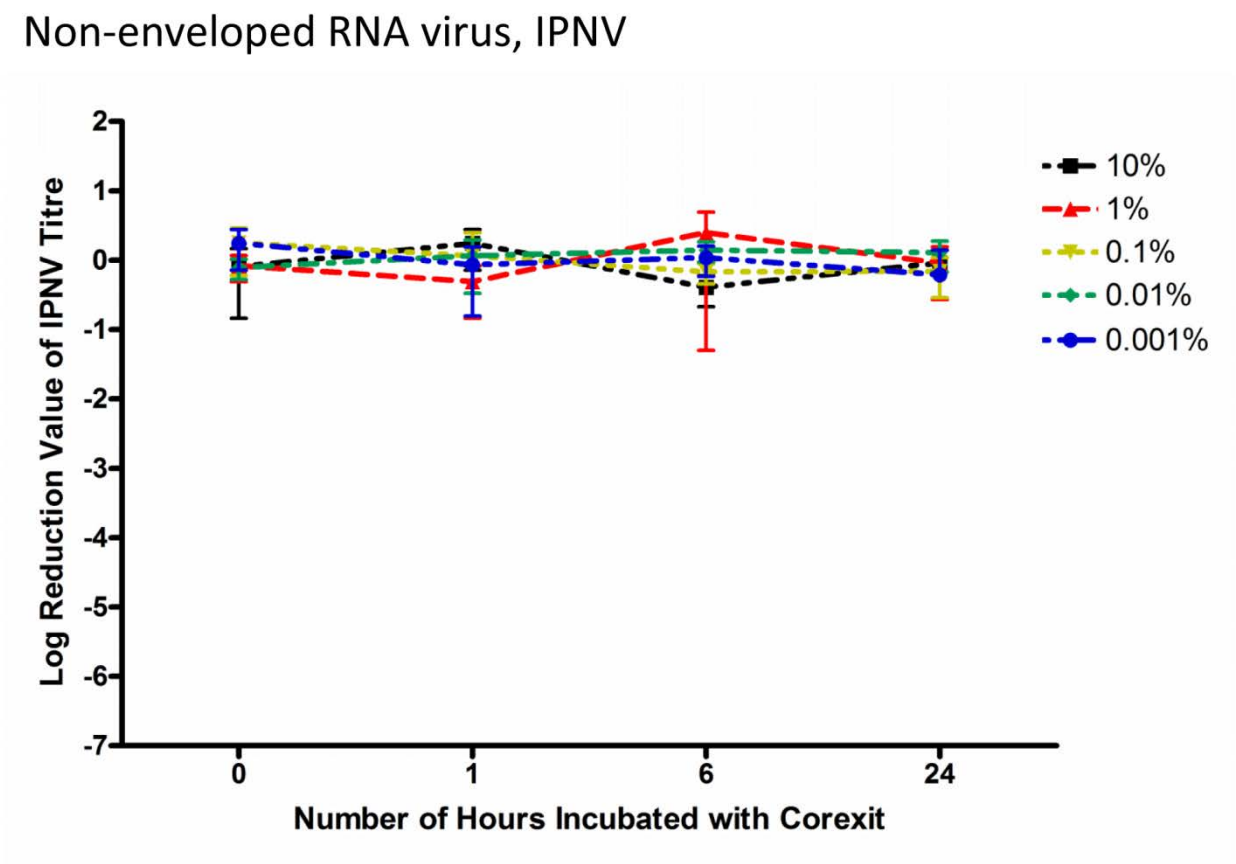


Fig. 5.2 Effect of Corexit 9500 on the titre of a Birnavirus, IPNV

IPNV was exposed to various dilutions of corexit 9500 in suspension tests over 24 h at 18 ^{o}C. As a control, the viruses were exposed to the same volume of DPBS in parallel. Immediately (time 0) and 1, 6, and 24 h after the Corexit exposure began, the mixture was sampled, the amount of virus was titred, and the log reduction value was calculated as described in the Materials and Methods section. The points on the graph represent means ± SD, n = 4.

5.3.4 Effect of Corexit 9500 on a virus with a single protein capsid and an envelope, VHSV

Exposure of VHSV IVb to dilutions of Corexit 9500 reduced the viral titre. At dispersant concentrations of 10% and 1%, VHSV was inactivated immediately after the virus was mixed with the dispersant at the 0 h time point, and at 0.1% complete inactivation was observed by 1 h post exposure (Fig. 5.3). At 0.01% concentration, a steady reduction in VHSV was observed over 6 h, with complete inactivation by 24 h (Fig. 5.3). However, at the most dilute concentration tested, 0.001%, reduction in VHSV titre was not observed up to the 6 h time point, but by 24 h, there was almost a 3 log reduction in viral titre when compared to control; however, complete inactivation was not observed (Fig. 5.3). At the two highest Corexit 9500 concentrations examined, 10% and 1%, virus titre was reduced beyond the lower limits of detection imposed by Corexit 9500 cytotoxicity; therefore, log reduction of virus can only be estimated by subtracting the virus titre with the lowest detectable titre (as defined in Section 5.3.1) and will be underestimated for these two concentrations. This is also the reason why Corexit 9500 at 0.1% concentration showed greater viral inactivation than 10% or 1% concentrations in the graph in Fig. 5.3.

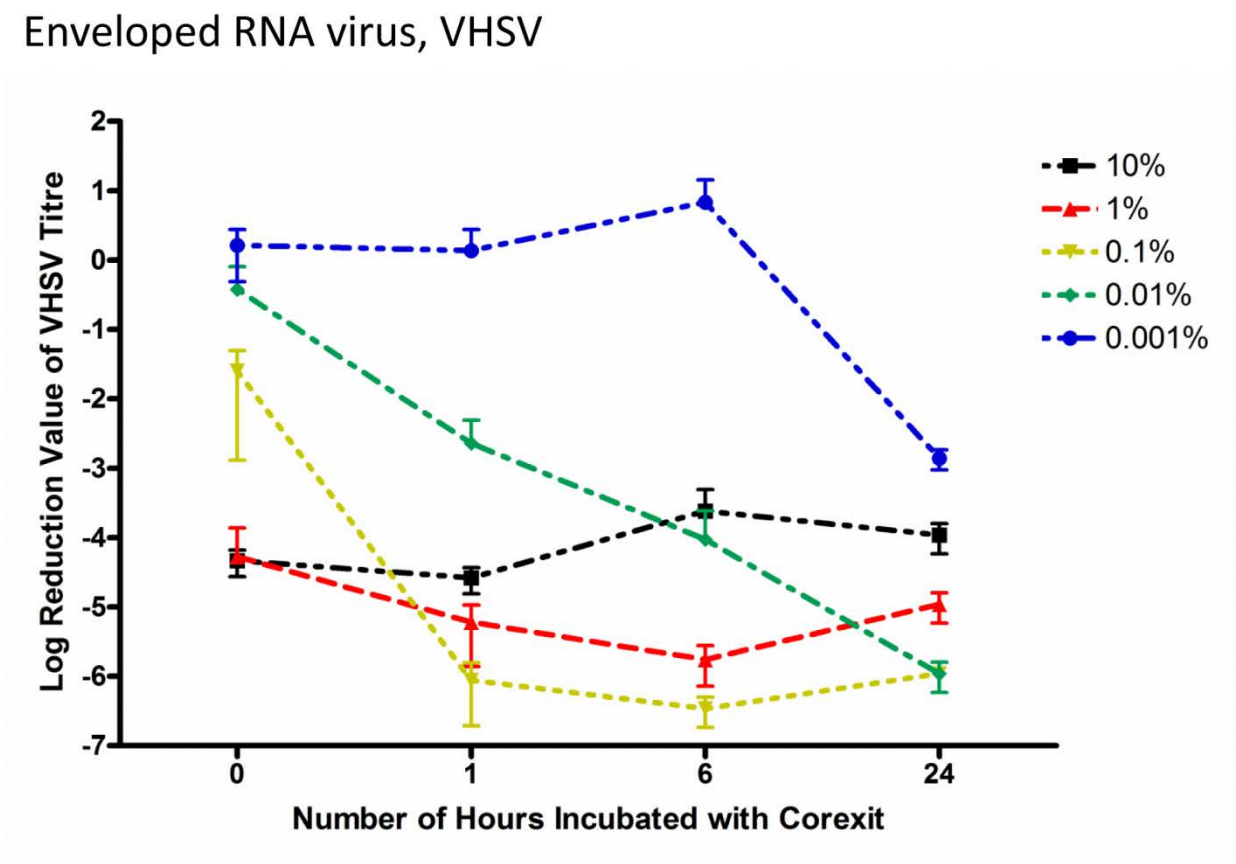


Fig. 5.3 Effect of Corexit 9500 on the titre of a Rhabdovirus, VHSV

VHSV IVb was exposed to various dilutions of corexit 9500 in suspension tests over 24 h at 14 ^{o}C. As a control, the viruses were exposed to the same volume of DPBS in parallel. Immediately (time 0) and 1, 6, and 24 h after the Corexit exposure began, the mixture was sampled, the amount of virus was titred, and the log reduction value was calculated as described in the Materials and Methods section. The points on the graph represent means ± SD, n = 4.

5.3.5 Effect of Corexit 9500 on a virus with a single protein capsid with or without an envelope, FV3

Exposure of FV3 to some concentrations of Corexit 9500 reduced the viral titre. At dispersant concentrations of 10% and 1%, FV3 was inactivated immediately after the virus was mixed with the dispersant at the 0 h time point (Fig. 5.4); this is similar to what was observed with VHSV in Fig. 5.3. At 0.1% concentration, reduction in FV3 titre was observed at the 1 h time point, but a slightly higher than 1 log reduction of FV3 titre was also observed at the 0 h time point (Fig. 5.4). However, at 0.01% and 0.001% FV3 was not inactivated by more than one log even after 24 h of exposure. Therefore, at more dilute concentrations of Corexit 9500, FV3 appeared to be more resistant to inactivation than VHSV in Fig. 5.3. Similar to the VHSV results, Corexit 9500 at 10% and 1% concentrations reduced FV3 titre to beyond the lower limits of detection; therefore, the reported log reduction values are underestimated for these two concentrations.

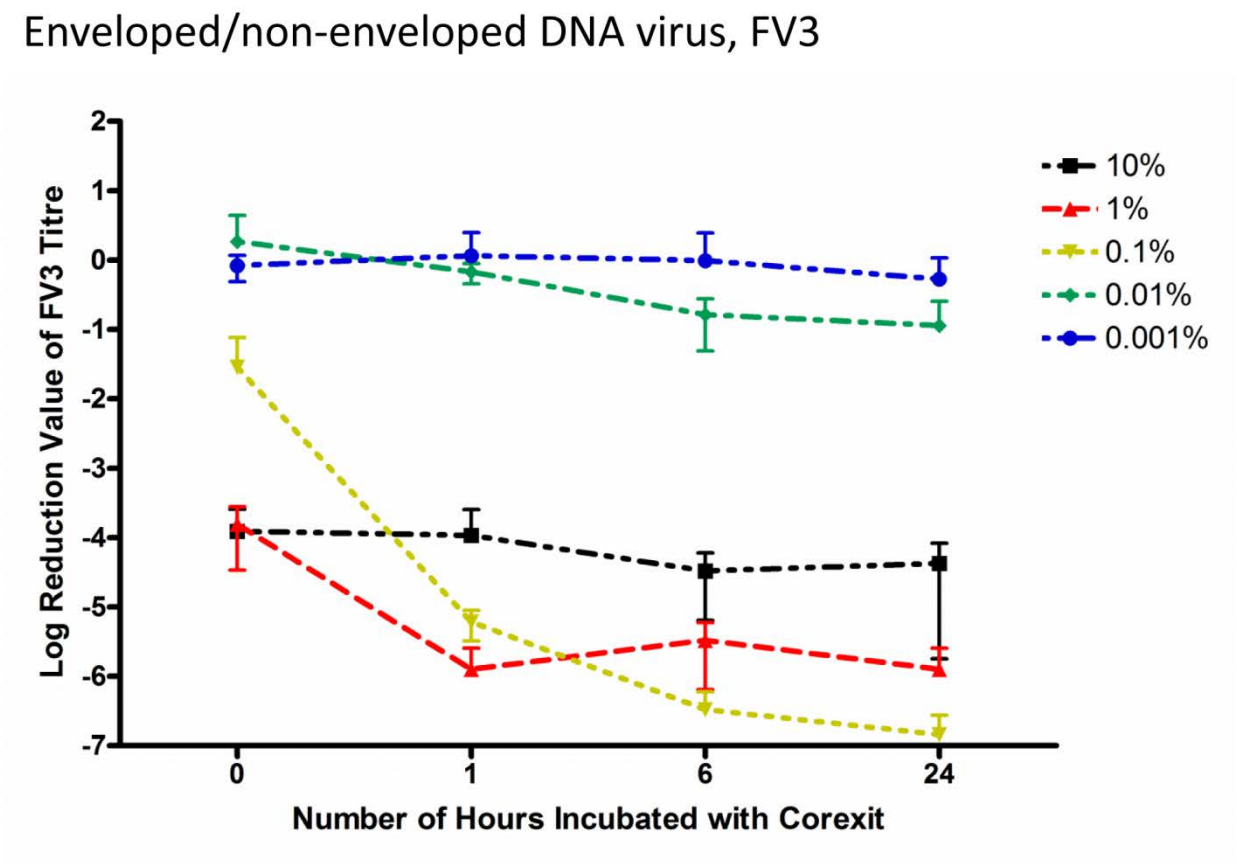


Fig. 5.4 Effect of Corexit 9500 on the titre of an Iridovirus, FV3

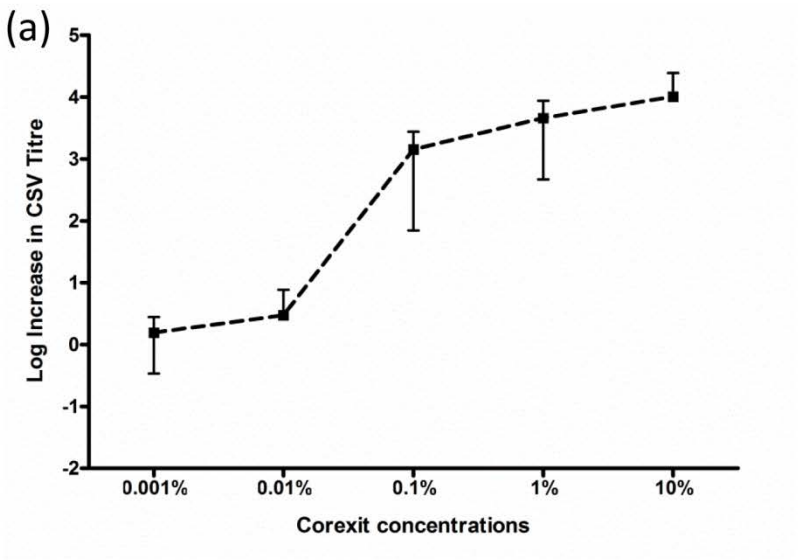
FV3 was exposed to various dilutions of corexit 9500 in suspension tests over 24 h at 20 ^{o}C. As a control, the viruses were exposed to the same volume of DPBS in parallel. Immediately (time 0) and 1, 6, and 24 h after the Corexit exposure began, the mixture was sampled, the amount of virus was titred, and the log reduction value was calculated as described in the Materials and Methods section. The points on the graph represent means ± SD, n = 4.

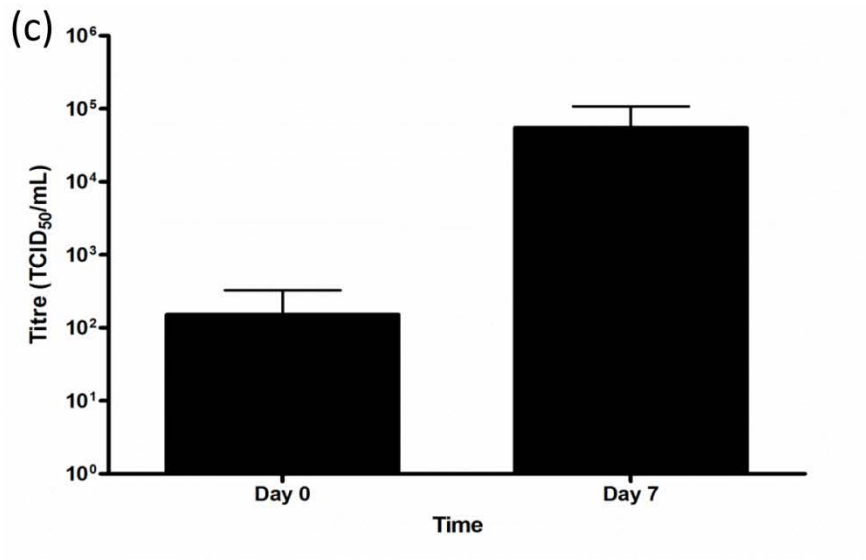
5.3.6 Effect of Corexit 9500 on a virus with a double protein capsid, CSV

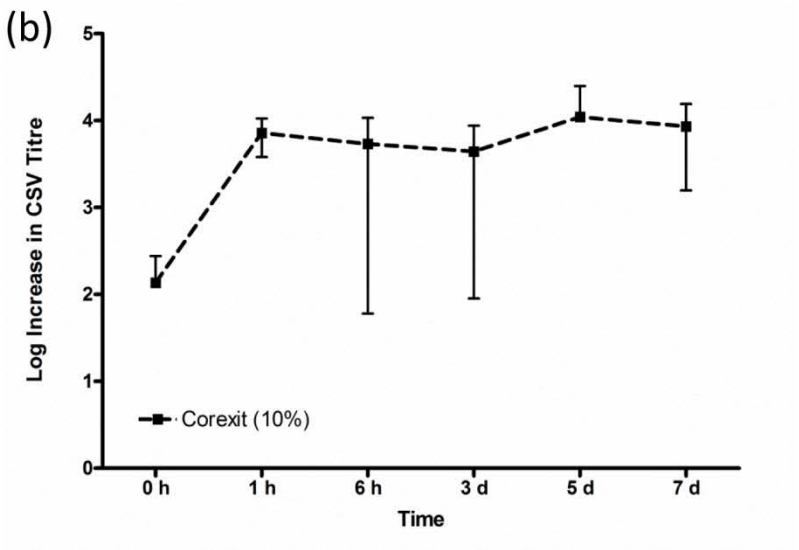
Exposure of CSV to Corexit 9500 produced a surprising result. The two lowest concentrations, 0.001% and 0.01% had no effect on CSV titre after 24 h of exposure (Fig. 5.5a), similar to IPNV in Fig. 5.2; however, at 0.1% concentration and higher, an increase of 3 to 4 logs in CSV titre was observed in Corexit 9500 exposed virus when compared to control virus exposed to only DPBS (Fig. 5.5a). Treatment of CSV with Corexit 9500 appeared to enhance the infectivity of the virus. To determine whether the virus would eventually be inactivated if kept in Corexit 9500 longer, CSV was exposed with Corexit 9500 at the highest concentration of 10% for 7 days; inactivation of CSV did not occur as the virus infectivity remained enhanced even after 7 days in the dispersant (Fig. 5.5b). Furthermore, increased in CSV titre, by approximately 2 logs, occurred immediately after the virus was mixed with the dispersant and maximal increase of nearly 4 logs occurred by 1 h (Fig. 5.5b). The increases in titre could be most straight forwardly explained by Corexit 9500 activating the CSV to a more infective form, referred to as Corexit enhanced CSV (eCSV).

Next, the ability of eCSV to produced infectious viral progenies was examined. CSV was mixed with Corexit 9500 at 10% final concentration for 24 h (the enhancement step); afterward, the mixture was serially diluted ten-fold to 10^{-4}. The 10^{-4} dilution was used to infect CHSE-214 cells and the titre produced was measured at day 0 and 7 post infection. The titre of CHSE-214 exposed to eCSV was observed to increase from approximately 10^{2} $TCID_{50}$/mL at day 0 to nearly 10^{5} $TCID_{50}$/mL by day 7 (Fig. 5.5c), similar to CHSE-214 cells infected with normal CSV (data not shown). The 10^{-4} dilution of eCSV was used instead of 10^{-1}, 10^{-2}, or 10^{-3} to avoid any possible toxic effect of Corexit 9500 on CHSE-214. Therefore, Corexit 9500 can enhance the infectivity of CSV and the eCSV can fully replicate and produce viral progenies. To determine whether the progeny virions produced from eCSV (day 7 in Fig. 5.5c) can also be enhanced, the virions were mixed with either DPBS or Corexit 9500 (10% final concentration) for 24 h before being titred on CHSE-214. Virions that were mixed with DPBS resulted in a titre of approximately 5.16×10^{4} $TCID_{50}$/mL while those that were mixed with Corexit 9500 showed a higher titre of 2.17×10^{6} $TCID_{50}$/mL (Fig. 5.5d). Therefore, Corexit 9500 can enhance the infectivity of CSV that had been produced from cultures initially infected with eCSV.

Non-enveloped RNA virus, CSV







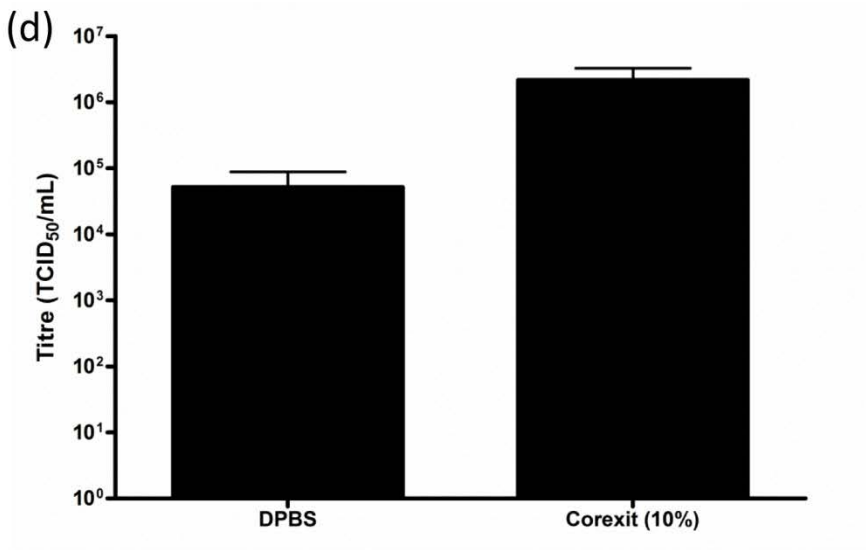


Fig. 5.5 Effect of Corexit 9500 on the titre of a Reovirus, CSV

In (a) the exposure of CSV to increasing concentrations of Corexit was done for 24 h at 18 °C using the same standard procedures and controls as described for the other viruses. The points on the graph represent means ±SD, n=4. In (b) CSV was incubated at a single high Corexit 9500 concentration of 10% for increasing times, for up to 7 days. At the 0 h time point the mixture was sampled for titre immediately after CSV and Corexit 9500 were mixed. Log increase values were calculated as described in the materials and methods section. The points on the graph represent means ±SD, n=4. In (c) CSV was treated for 24 h with Corexit 9500 (10% concentration), which is referred to as eCSV. The virus/Corexit 9500 mixture was diluted to 10-4 and added to CHSE-214 cultures. The virus titre produced from the cultures receiving the 10-4 eCSV was measured at day 0 and day 7 post infection (PI). The points on the graph represent means ±SD, n=6. In (d), the progeny virions of eCSV that was collected at day 7 PI in (c) were exposed to either DPBS or Corexit 9500 (10% concentration) for 24 h before titration on CHSE-214 to determine if the infectivity of progeny virions of eCSV can also be enhanced by Corexit 9500. The points on the graph represent means ± SD, n = 3.

5.4 Discussion

The effects of Corexit 9500 on four aquatic viruses of differing structural properties were examined. Corexit 9500 was able to significantly inactivate the viruses with envelopes, VHSV and FV3, but had no effect on the non-enveloped virus, IPNV. By contrast, Corexit 9500 activated the virus with a double protein capsid, CSV. Although the complete formulation of Corexit 9500 is not available, the components include alcohols, such as 1-propanol, and nonionic and anionic surfactants which are used in detergents [3-5,23]. The interaction of these components with the four viruses is discussed below, followed by a consideration of the environmental implications of the results.

5.4.1 Effect of Corexit 9500 on a virus with a single protein capsid, IPNV

Corexit 9500 at all concentrations examined, did not significantly reduce IPNV titre. Relative to enveloped viruses, naked viruses have generally been found to be more resistant to inactivation by alcohol-based disinfectants [24-26]. In addition, the surfactant, Sorbitan, mono-(9Z)-9-octadecenoate, poly(oxy-1,2-ethanediyl), a component of Corexit 9500 [23] and a major component of Tween 80, had been previously demonstrated to enhance non-enveloped poliovirus yield instead of reducing titre [27].

5.4.2 Effect of Corexit 9500 on a virus with a single protein capsid and an envelope, VHSV

The inactivation of VHSV IVb by Corexit 9500 is likely mediated through the components of the dispersant, such as alcohols and surfactants, acting on the lipids and proteins of the viral envelope because these classes of compounds have been found to inactivate other enveloped viruses. Several studies found alcohols inactivated Rhabdoviruses, including one from mammals, vesicular stomatitis virus (VSV), as well as VHSV. 1-propanol at concentrations of 40% or higher abolished the infectivity of VHSV absorbed onto polystyrene surfaces [26]. Ethanol, 1-propanol and 2-propanol at less than 50% concentrations were shown to reduce VSV titre by greater than 6 logs after 1 min of exposure in suspension tests [28]; at higher concentrations, 75% of combined 1-propanol and 2-propanol and 80% or higher ethanol, can reduce the titre of six different

mammalian enveloped viruses by greater than 4 logs within 15 s of contact time [29]. The lipid membrane of enveloped VHSV and FV3 could undergo structural disorder upon exposure to alcohols [30] and this change in membrane structure may prevent proper exposure of viral surface proteins, such as the glycoprotein of VHSV, blocking interaction with cellular receptors and viral entry into the cells. Surfactants also have a significant inactivating effect on enveloped viruses. Less than 1% concentrations of non-ionic Triton X-100 and anionic sodium dodecyl sulfate (SDS) reduced VSV titre by greater than 6 logs after 5 min of exposure in suspension tests [28]. Benzalkonium chloride, a cationic surfactant, at 1% or lower concentration can reduce the titre of enveloped herpes simplex virus and human immunodeficiency virus by 4.51 and 1.87 logs, respectively after 1 min of exposure [25]. The mechanism of reduction in enveloped virus titre by surfactants is most likely due to solubilization of membrane lipids, releasing membrane bound proteins, separating these components from the core of the virus and preventing the delivery of the viral genome into cells [31].

5.4.3 Effect of Corexit 9500 on a virus with a single protein capsid with or without an envelope, FV3

Surfactants and alcohols in Corexit 9500 likely inactivated FV3 also by targeting the envelope but FV3 was more resistant than VHSV to inactivation. Surfactants have been found to inactivate at least one other *Ranavirus*. The titre of Singapore grouper iridovirus (SGIV) was reduced by approximately 2 logs when treated in suspension tests for 1 h with three different surfactants: 0.1% Triton X-100, and 0.08 mM of n-octyl-β-D-glucopyranoside (OG) and n-dodecyl-α-D maltoside (DDM) [32]. Corexit 9500 at dilutions of 10^{-4} and 10^{-5} had a less inactivating effect on FV3 than on VHSV. This could be due to the simultaneous existence of two virion populations in FV3, both enveloped and non-enveloped virions; the reduction in FV3 titre is likely due to inactivation of enveloped virions as they are also more infectious than non-enveloped virions [33]. As well as mentioned with IPNV, Corexit 9500 had no effect on a virus with just a capsid.

5.4.4 Effect of Corexit 9500 on a virus with a double protein capsid, CSV

Two mechanisms, working independently or synergistically, likely explain how Corexit 9500 enhanced the infectivity of CSV. One mechanism revolves around aggregation: the dispersant either prevented aggregation or broke apart aggregates of CSV. Reoviruses can aggregate, especially in solutions with pH 5 or lower [34]. The dissociation of aggregated virus particles had been shown to increase viral titre in cell culture [35] and detergents can disperse aggregated viruses [36]. The second mechanism involves the second capsid of Reoviruses. Corexit 9500 exposure could be removing the outermost capsid of CSV, generating infectious subviral particles (ISVP). Treatment of Reoviruses, including CSV, with proteases such as trypsin and chymotrypsin, generates a smaller ISVP, through digestion of the outermost capsid protein, that lead to an enhancement in the infectivity of these viruses [37-39]. Therefore, Corexit 9500 may act in a duel manner to enhance CSV infectivity.

5.4.5 Corexit 9500 and the virosphere

The results suggest that oil dispersants could modulate the virosphere and in doing so alter fundamental ecological processes, which in turn could have broad environmental impacts. Corexit 9500 inactivated the two enveloped viruses, but left the non-enveloped viruses either unharmed or activated. The sea likely has enveloped viruses as well as non-enveloped viruses. Most aquatic bacteriophages are non-enveloped but the ocean also appears to be abundant with RNA viruses that infect eukaryotes [40]. Some of these are enveloped [16]. If the pattern of results from this study with four fish viruses extends to all viruses, the addition of Corexit 9500 to an aquatic environment might be expected to reduce enveloped viruses while allowing non-enveloped viruses to endure or be activated. Changing viral assemblages in this way might impede or enhance the important roles of viruses in ecological processes, such as the microbial loop, and change the environment. On the other hand, altered viral assemblages would likely be transitory because the sheer number of viral particles in the sea would allow some to remain and be capable of restoring the original viral populations, and of course, the use of dispersants would be temporary. Exploring these ideas in the future would be interesting but likely daunting because of the diversity of viruses and the complexity of ecological interactions.

For viruses of metazoans, the actions of oil dispersants might influence the prevalence of viral diseases. The inactivation of VHSV by Corexit 9500 is particularly intriguing as some of the most important viral diseases of fish are caused by Rhabdoviruses [41]. Situations can be envisioned where Corexit 9500 might be suggested as a way to halt the spread of economically damaging diseases like viral haemorrhagic septicaemia and infectious hematopoietic necrosis. However, the activation of CSV by this dispersant would be one virology argument against such a use.

CHAPTER 6

General conclusions

The overarching goal of this thesis was to examine factors affecting the transmission of aquatic viruses: these factors are the abilty of viruses to infect cells at the cellular level, to upregulate cellular immune responses, and to persist in the environment. These factors can determine the ability of organisms to become hosts for viruses. More specifically, to be a host, organisms must come into contact with viruses and allow viruses to sucessfully enter cells and replicate. Entry and replication of aquatic viruses in many fish cell lines of different fish and cell types was examined in Chapters 2, 3 and 4, using four model aquatic viruses VHSV genotype-IVa, -IVb, GIV and FV3, and the persistence of aquatic viruses of differing structural composition in Corexit 9500 contaminated environment was examined in Chapter 5.

Virus entry and replication in fish cell lines was experimentally determined by infecting the cell lines with the viruses and looking for the ability of viruses to cause cytopathic effect (CPE), to express viral genes (indicating virus entry into cells) and to successfully replicate by producing virions. The interactions between the viruses (VHSV genotype-IVa, -IVb, GIV and FV3) and cell lines examined in this thesis are summarized in Table 6.1.

From Table 6.1, brief global cross comparison and general conclusions can be made. In terms of CPE, VHSV-IVa and IVb induced cell death in RTgill-W1 but not RTS11. GIV induced cell death in all infected non-host cell lines by apoptosis. All rainbow trout cell lines examined in this thesis were susceptible to FV3 induced cell death but only RTS11 died as a result of apoptosis. In terms of virus entry, each virus was able to enter and initiate the viral replication cycle because at least some viral genes were expressed in the various cell lines. The last common theme examined was the ability of the viruses to replicate in the cell lines. In this case, the results were quite different for each of the virus-cell line interactions. RTS11, the monocyte/macrophage cell line, was unable to support the replication of any of the viruses examined. RTgill-W1 was able to support VHSV-IVa and –IVb replication but not FV3. Similary, most rainbow trout cell lines was unable to support FV3 replication but EPC can support both the replication of GIV and FV3. Following this brief cross comparion is the detailed summary, anaylsis and discussion of the results in Table 6.1 and Chapter 5.

Table 6.1 Comparison of the response of fish cell lines to aquatic viruses

Cell lines	VHSV IVa	VHSV IVb	GIV	FV3
Summary of CPE observed in cell lines				
GK	ND	ND	+	ND
CHSE-214	ND	ND	+ (apoptosis)	ND
EPC	ND	ND	+ (apoptosis)	+
RTS11	-	-	+ (apoptosis)	+ (apoptosis)
RTgill-W1	+	+	ND	+
RTG-2	ND	ND	ND	+
RTgutGC	ND	ND	ND	+
RTL-W1	ND	ND	ND	+
RTHDF	ND	ND	ND	+
Summary of viral gene expression in cell lines (virus entry into cells)				
GK	ND	ND	+	ND
CHSE-214	ND	ND	+ (early and late genes)	ND
EPC	ND	ND	+ (early and late genes)	+ (late gene)
RTS11	+		+ (early genes only)	+ (late gene)
RTgill-W1	+		ND	+ (late gene)
RTG-2	ND	ND	ND	+ (late gene)
RTgutGC	ND	ND	ND	+ (late gene)
RTL-W1	ND	ND	ND	+ (late gene)
RTHDF	ND	ND	ND	+ (late gene)
Summary of virus titre produced in cell lines (successful virus replication)				
GK	ND	ND	High	ND
CHSE-214	ND	ND	-	ND
EPC	ND	ND	Moderate	High
RTS11	-	-	-	-
RTgill-W1	High	Moderate	ND	-
RTG-2	ND	ND	ND	Moderate
RTgutGC	ND	ND	ND	-
RTL-W1	ND	ND	ND	Low
RTHDF	ND	ND	ND	-

Note 1: + positive CPE or viral gene expression

Note 2: - negative for CPE, increase in virus titre or viral gene expression

Note 3: ND – No data presented in thesis

6.1 Comparing the responses of rainbow trout gill and macrophage cells to VHSV genotype IVa and IVb

Both VHSV genotypes VHSV IVb and IVa were able to infect and replicate in the epithelial cell line, RTgill-W1, but replication did not occur in the monocyte/macrophage cell line, RTS11. Although innate antiviral genes were upregulated by virus infections in RTgill-W1, the infections resulted in the eventual death of the cells. In RTgill-W1 VHSV genotype IVa was more infectious than VHSV IVb; VHSV IVa caused CPE earlier and at a much lower titre and produced a higher amount of viral progenies. The infection of RTS11 cultures with VHSV IVa and IVb produced a different outcome: no CPE and no increase in viral titre were seen. Yet both viral strains did enter RTS11 and expressed viral genes but viral proteins were not detected. However, innate antiviral genes were upregulated in RTS11. Therefore, the replication cycle of VHSV IVa and IVb in RTS11 appeared to be terminated at the translational stage. A graphical summary of the response of RTgill-W1 and RTS11 to VHSV IVa and IVb is shown in Fig. 6.1. The ability of VHSV IVa and IVb to infect gill cells suggests that the gills are potential portals of entry for the virus into the organism, which can lead to viral transmission. In this case, macrophages are good early responder to VHSV because of their ability to soak up the virus, abort the infection and at the same time, enter into an antiviral state and further prime the immune system.

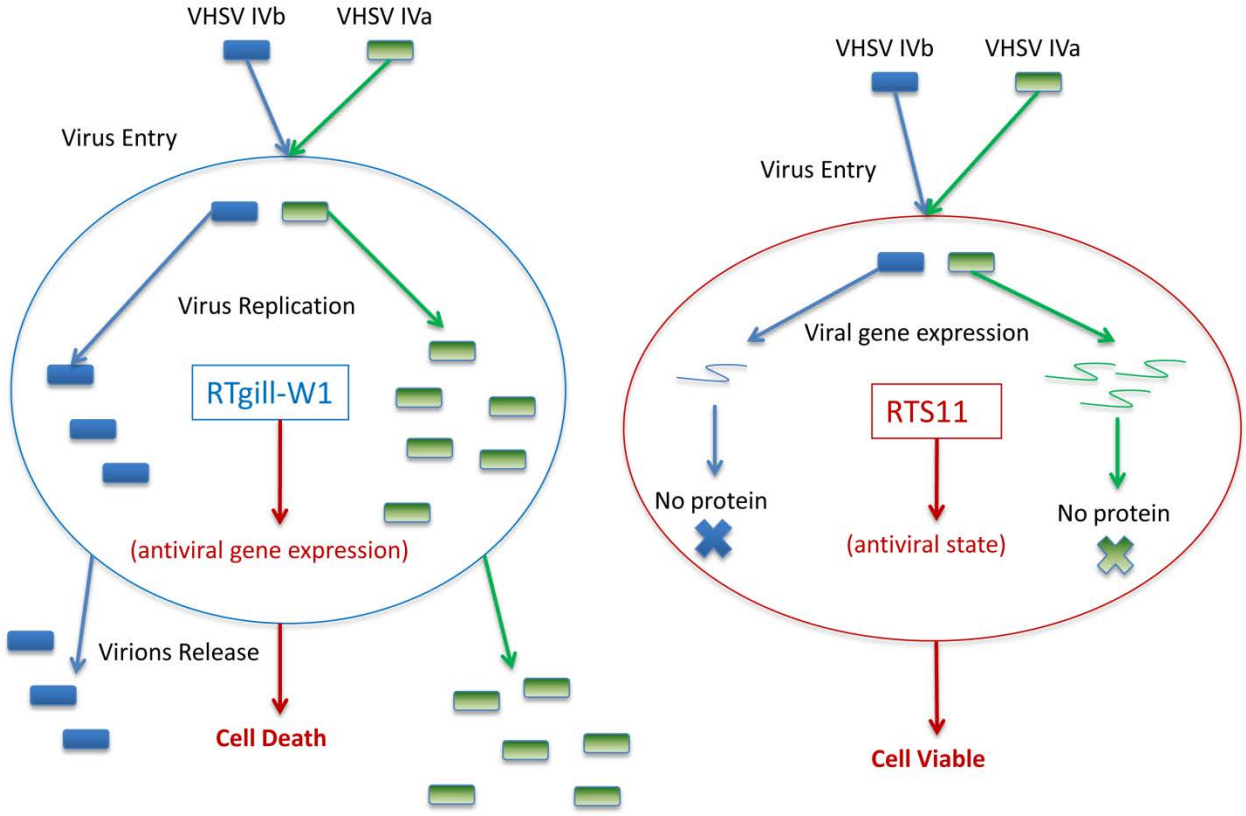


Fig. 6.1 Response of RTgill-W1 and RTS11 to VHSV IVa and IVb

VHSV IVa and IVb entered into RTgill-W1 and fully replicated to produce infectious virions. Infection of RTgill-W1 cells led to upregulation of antiviral genes but eventually led to cell death. In contrast, VHSV IVa and IVb infections of RTS11 were aborted at the translational stage. Although VHSV transcripts were detected, the normally most abundant N protein was not. RTS11 entered an antiviral state in response to the infection by expressing antiviral genes.

6.2 Comparing the responses of cell lines to GIV and FV3

The ability of non-host fish cell lines to support the infection of GIV and FV3 was examined to determine the potential role of cellular tropism in virus host shifts. GIV was able to induce apoptotic cell death in EPC, CHSE-214 and RTS11; however, the events leading to the induction of cell death for each cell line was different. By studying the interaction of GIV with these three different cell lines, three possible scenarios for virus-host cell interaction is hypothesized. These are discussed below and visualized in Fig. 6.2. When GIV entered RTS11, apoptosis was induced early during the infection, before expression of late major capsid protein (MCP) gene, possibly initiated by virion proteins. When GIV entered CHSE-214, cell death occurred after the MCP gene was expressed; in addition, active viral gene expression is required for induction of apoptosis in CHSE-214 because UV irradiated virus did not cause cell death. This suggests that the factor responsible for the induction of cell death was an expressed viral gene, possibly pro-apoptotic genes. GIV successfully replicated in EPC; however, successful replication is believed to require early expression of viral anti-apoptotic genes to delay cell death and allow enough time for the virus to complete its replication cycle; the evidence for this conclusion comes from the result that UV irradiated virus enhanced apoptosis. Additionally, GIV had been previously shown to carry a homologue of the anti-apoptotic Bcl-2 gene [1]. Late in the virus replication cycle, apoptosis is induced in EPC, allowing the release of virions.

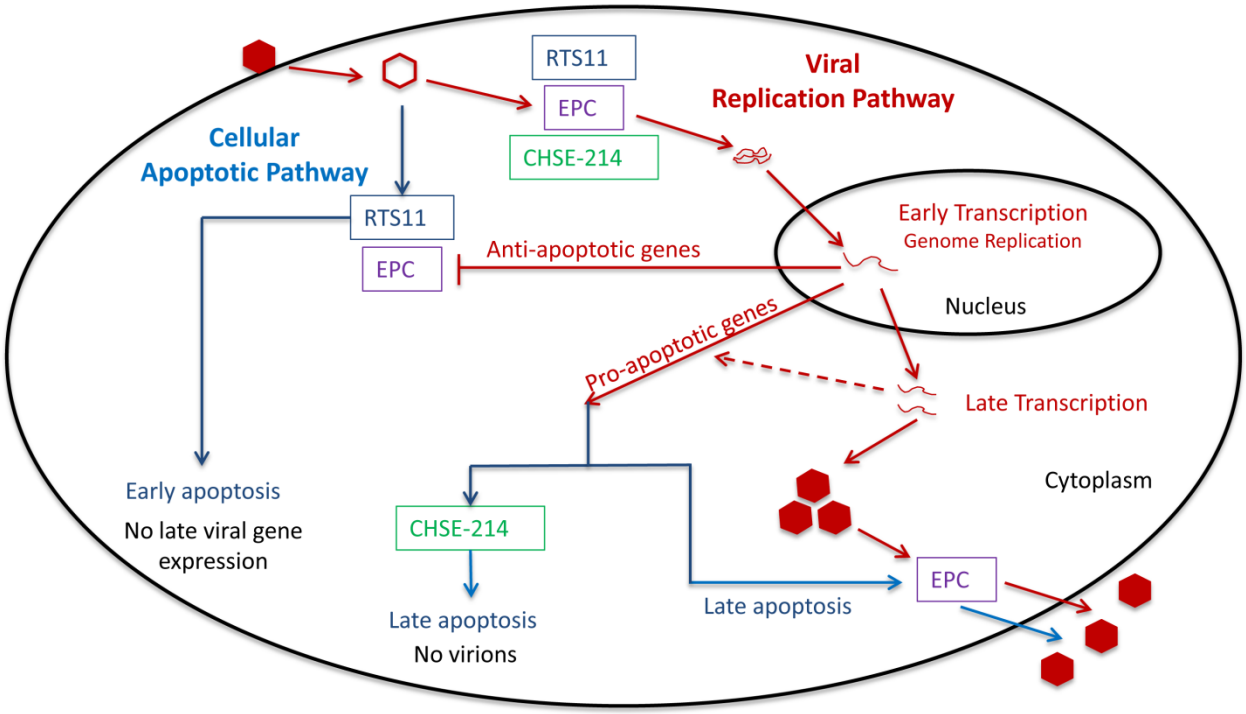


Fig. 6.2 Three hypothetical scenarios for the interaction of GIV with RTS11, EPC, and CHSE-214

Detailed descriptions of the interaction can be found in text. Briefly, RTS11 is believed to undergo apoptosis upon infection by GIV before the virus entered the late stage of the replication cycle. Induction of apoptosis in CHSE-214 by GIV was dependent on viral gene expression and occured after the virus entered the late stage of the replication cycle. GIV infected and fully replicated in EPC cells and apoptosis was induced after virions were fully formed to aid in the release of the virus.

The infection of the same fish cell lines with FV3 and GIV showed both similarities and differences in outcomes (Table 6.1). Both viruses induced apoptosis in RTS11; however, the MCP gene was expressed only by FV3 in RTS11. In addition, FV3 did not induce early apoptosis in EPC but GIV did. The differences in the interaction of GIV and FV3 with EPC could possibility be attributed to the large difference in their genome sequence identity and the number of proteins or putative proteins express by each virus. Although both GIV and FV3 are from the same genus, they formed separate clades at the species level on a phylogenetic tree, and nucleotide sequence analysis of the essential MCP genes only showed approximately 71% sequence identity between the two species [2]. In addition, the FV3 genome has approximately 97 Open Reading Frames (ORF) or putative proteins while the GIV genome has approximately 139 ORF [3], allowing for possibly more GIV proteins to interact with host cellular machinery. Therefore it may not be surprising that there was a difference in the cellular responses to these viruses; furthermore, the induction of early apoptosis in EPC offers a potential diagnostic tool to differentiate between *Ranavirus* species.

FV3 infections of rainbow trout cell lines from different anatomical sites resulted in cell death for all the cell lines but only the monocyte/macrophage RTS11 died by apoptosis. Similarly, FV3 infections of human lymphocytic PEER cell line did not result in cell death but the virus caused apoptosis in the monocytic U937 cell line. These experiments showed that cells of the monocytic lineage were more sensitive to *Ranavirus* induced apoptosis than other cell types. The mechanism may be related to the known ability of FV3 to significantly inhibit host cell DNA, RNA and protein synthesis [16,17] combined with the high sensitivity of monocytic cells to apoptosis resulting from inhibition of cellular macromolecule synthesis. A previous comparison of the response of RTS11, RTG-2 and CHSE-214 to transcriptional inhibitor actinomycin D and translational inhibitor cycloheximide showed that these two agents more readily induce apoptosis in RTS11 than the other two cell lines [4]. Similarly, human fibroblastic L929 cells and lymphocytes were much more resistant to cell death induced by actinomycin D and cycloheximide than human macrophages [5].

At the cellular level rainbow trout cells appeared refractory to FV3 infections. The minimal ability of FV3 to replicate in rainbow trout cells suggests that cells are one

barrier to the establishment of FV3 infections in rainbow trout. If *in vivo* infections of rainbow trout cells with FV3 are similar to *in vitro* results, then the lack of significant virion production means that the virus may not replicate enough to overwhelm and circumvent the immune defenses of rainbow trout.

6.3 Comparing the responses of macrophages to viruses of *Rhabdoviridae* and *Iridoviridae*

The effect of viruses on macrophages, particularly the monocyte/macrophage cell line RTS11, is a common theme throughout this thesis. Although the responses of RTS11 to viruses of the *Rhabdoviridae* family differed from those of the *Iridoviridae* family, viruses in both families underwent aborted infections in RTS11. With VHSV genotype IVa and IVb of *Rhabdoviridae* family, infections of RTS11 were aborted but the cells remained viable. With GIV and FV3 of the *Iridoviridae* family, infections were also aborted but as a result of RTS11 undergoing apoptosis. In the case of VHSV, the inability of the virus to infect macrophages may be the result of adaptations in the host defense system against this family of viruses. Macrophages can activate the innate immune system and can also participate in antigen presentation of adaptive immune system; therefore, keeping macrophages alive ensured a strong and complete defense against Rhabdovirus infections [6-8]. However, in contrast, the induction of apoptosis in macrophages by GIV and FV3 could be a response elicited by the virus to weaken the host innate immune system. In frogs, tadpoles are more susceptible to FV3 infection than adults, likely due to an incompletely developed immune system; tadpoles rely more on innate immunity for defense against infections [9]. Therefore, elimination of macrophages by FV3 or *Ranaviruses* in general would greatly favour the establishment of infections in the more susceptible life stage of frogs. In addition to removing an important cell type of both the innate and adaptive immune responses, the mode of cell death also favored evasion of the immune system. Death by apoptosis, as opposed to necrosis, leads to suppression of the inflammatory response in the host and reduces the activation of the immune system [10].

6.4 Comparing the responses of enveloped and non-enveloped viruses to Corexit 9500

Enveloped viruses such as VHSV and the enveloped virions of FV3 were more sensitive to Corexit 9500 inactivation than non-enveloped viruses like IPNV and CSV. The hypothesized mechanism involves the detergent component of Corexit 9500 solubilizing the virus envelop [11] and/or the alcohol components disordering membrane structure [12]. Corexit 9500 was unable to significantly inactivate IPNV and this finding is supported by previously published results showing the generally higher resistant of non-enveloped virus to inactivation by disinfectants than enveloped virus [13-15]. The most surprising result was the enhancement of CSV infectivity by Corexit 9500, which may be attributed mechanistically to the separation of virus aggregates or the removal of the outer protein capsid of CSV, creating more infectious subviral particles (ISVP). The results suggest that enveloped viruses in the aquatic environment are sensitive to Corexit 9500 inactivation and the use of dispersants may at least reduce the ecological role of enveloped viruses in the environment.

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