A new angle on plastic debris in the aquatic environment: Investigating interactions between viral hemorrhagic septicemia virus (VHSV) and inanimate surfaces

by

Phuc Hoang Pham

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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

Methods of studying the interaction between fish viruses with inanimate surfaces were developed and used to explore several variables. Viral hemorrhagic septicemia virus (VHSV) was used as the model virus. The EPC cell line, which is now known to be from Fathead Minnow, was used to detect the virus through the development of cytopathic effect (CPE); this allowed virus levels to be titrated and expressed as tissue culture infectious dose ($TCID_{50}$). The labour and tedium of scoring hundreds of wells for CPE was overcome through the use of the fluorescent indicator dye, Alamar Blue, which is reduced by living cells and not by dead cells to yield a fluorescent product that can be measured as relative fluorescence units (RFUs) with a fluorescent microwell plate reader. Microsoft Excel 2007 was used to compare RFU values of wells and to create a scoring template in the computer program that allowed for easy summation of the total number of wells with infectious virus. With this system and as well as with conventional scoring, surface-virus interactions were studied in the following general way. Surfaces were incubated with a solution (L-15 with 2% fetal bovine serum or FBS) of virus, rinsed, and then incubated under various conditions, either wet or dry, before being evaluated for infectious virus.

The transfer of viruses through their elution from surfaces is termed elution transfer (ET) and was investigated in two ways: agitated elution and static elution. Agitated elution was done through the repeated action of pipetting up and down on either glass or plastic surfaces with different eluting solutions. The best eluting solution was 2% FBS/L-15 and the worst was tissue culture grade water. Regardless of the eluting solution, no infectious virus could be removed by agitated elution from glass Petri dishes. Static elution was demonstrated through a two-compartment culture system linked by 3.0 μm pores. L-15 with 2% FBS eluted VHSV from the surface of the top chamber to infect cells in the bottom chamber and from the surface of the bottom chamber to infect cells in the top chamber.

The ability of different objects to carry infectious VHSV to a new culture vessel was investigated in a protocol termed object-associated transfer (OAT). The objects were incubated with VHSV, rinsed, and then incubated wet or dry for various periods before being transferred to EPC cultures. After up to ten days of wet incubation, pieces of glass, fishing line, plastic water bottle, and pop can were able to transfer infectious virus. In contrast, when the same objects were incubated dry, they were able to transfer VHSV after only one day of drying. Fishing hooks kept wet for a day were able to transfer VHSV but dry hooks had no capacity to transfer infectious virus.

A third experimental protocol was used to detect infectious viruses on surfaces and involves the surface to cell transfer (SCT) of viruses. For this protocol, EPC cells were plated directly onto plastic or glass surfaces that previously had been exposed to virus, rinsed, and incubated dry or wet at various temperatures for up to 15 days. After 15 days being kept dry at 4 °C, infectious VHSV was still found to be present on both glass and plastic surfaces. At 14 °C and room temperature, the virus was found to survive longer on plastic than on glass, and at 26 °C both surfaces retained infectious VHSV for only one day of being dry. Survival time on plastic surfaces at different temperatures was compared for wet and dry incubation. VHSV kept on plastic surface in a dry state was more susceptible to temperature inactivation, with inactivation of the virus being detected clearly after 1 day 37 °C, 10 days at 26 °C, and 15 days at room temperature.

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DEDICATION

I dedicate this thesis to my sister, parents and grandparents. Thank you for your faith and support.

Table of Contents

LIST OF FIGURES ..... **x**

LIST OF TABLES ..... **xii**

LIST OF ABBREVIATIONS ..... **xiii**

CHAPTER 1 INTRODUCTION **1**

1.1 Anthropogenic debris in the aquatic environment 1

1.2 The interaction between fomites and viruses 3

1.3 Fish viruses 4

1.4 The interaction between fomites and viruses 4

1.4.1 Physical characteristics and identification of VHSV 5

1.4.2 Molecular properties and phylogenetic of VHSV 5

1.5 Environmental factors contributing to the inactivation of microorganisms and viruses 6

1.5.1 Open air factor and ozone 6

1.5.2 Ultraviolet radiation 7

1.5.3 Relative humidity and temperature 8

1.6 Survival and/or inactivation of VHSV 8

1.7 Factors that influence viral attachment to and survival on inanimate surfaces 9

CHAPTER 2 MATERIALS AND METHODS **11**

2.1 Cell culture 11

2.2 Virus propagation and identification 11

2.3 Determining tissue culture infectious dose ($TCID_{50}$) of VHSV using the Karber method 11

2.4 Scoring $TCID_{50}$ plates with Alamar Blue (AB) assay 13

2.5 Detection of VHSV attachment and survival dried on various surfaces using the agitated elution transfer (ET) method 13

2.6 Detection of VHSV attachment and survival dried on PET track-etched membrane cell culture insert using the static elution method 16

2.7 Detection of VHSV attachment and survival dried on various potential fomites using the object-associated transfer (OAT) method 16

2.8 Detection of VHSV attachment and survival in dry and wet conditions at different temperatures on various 60 mm dishes using the surface-to-cell transfer (SCT) method 20

2.9 Relative quantification of VHSV attachment and survival in dry and wet conditions at various temperatures on 96-well microtiter plates using the surface-to-cell transfer (SCT) method 22

CHAPTER 3 RESULTS **26**
3.1 Comparison of $TCID_{50}$ values derived from visual scoring vs. AB scoring of $TCID_{50}$ plates 26
3.2 Examining VHSV attachment and survival dried on various 60 mm dish surfaces and PET track-etched membrane using the elution transfer (ET) method 28
3.2.1 Agitated elution 28
3.2.2 Static elution 30
3.3 Examining VHSV attachment and survival on various objects using the object-associated transfer method 32
3.3.1 Soda lime glass beads 32
3.3.2 Fishing Hooks 35
3.3.3 Fishing lines 38
3.3.4 Commercial soft drink can and water bottle pieces 40
3.4 Examining VHSV attachment and survival on various objects using the surface-to-cell transfer (SCT) method 42
3.4.1 The Survival of VHSV left to dry on 60 mm plastic dishes at various temperatures ... 44
3.4.2 The survival of VHSV left to dry on 60 mm glass dishes at various temperatures 47
3.5 Relative quantitative comparison of VHSV inactivation using 96-well microtiter plate SCT method 49
3.5.1 Relative quantitative comparison of VHSV survival at 4°C in dry and wet environments 52
3.5.2 Relative quantitative comparison of VHSV inactivation at 14°C in dry and wet environments 54
3.5.3 Relative quantitative comparison of VHSV inactivation at room temperature in dry and wet environments 56
3.5.4 Relative quantitative comparison of VHSV inactivation at 26°C in dry and wet environments 58
3.5.5 Relative quantitative comparison of VHSV inactivation at 37°C in dry and wet environments 58
3.5.6 Comparison of VHSV inactivation across all incubation temperatures in dry and wet conditions 61
CHAPTER 4 DISCUSSION **63**

4.1 Rapid scoring of multiwell plates for infectious VHSV using Alamar Blue and Microsoft Excel 2007 63
4.2 Examining the elution transfer (ET) method of VHSV 64
4.2.1 The events from attachment of VHSV to appearance of CPE on reporter cells 64
4.2.2 The attachment of VHSV to surfaces 65
4.2.3 The survival and inactivation of VHSV dried on various surfaces 66
4.2.4 The role of various solutions in the elution of dried VHSV from various surfaces 66
4.2.5 The potential inactivation of VHSV during and after elution 68
4.3 Examining the object associated transfer (OAT) method of VHSV 69
4.3.1 Wet Object-associated transfer of VHSV 69
4.3.2 Dry object-associated transfer of VHSV 70
4.4 Examining the surface-to-cell transfer (SCT) method of VHSV 73
4.4.1 Mechanism of surface-to-cell transfer of viruses 73
4.4.2 Comparing surface-to-cell transfer method with elution transfer method 75
4.4.3 Using SCT method to study the effect of temperature on VHSV survival in wet and dry environments 76
4.5 Possible role of floating debris in transporting fish viruses in the aquatic environment 78
4.6 Possible role of floating debris in transmitting viruses to fish 80
APPENDICES **82**
Appendix A **82**
Appendix B **83**
Appendix C **85**
REFERENCES **88**

LIST OF FIGURES

Figure 1- 1. TCID50 setup on a 96-well microtiter plate. 12
Figure 1- 2. Schematic diagram for the agitated elution transfer (ET) method. 15
Figure 1- 3. Schematic diagram for the object-associated transfer (OAT) method involving glass beads in dry condition. 19
Figure 1- 4. Schematic diagram for the surface-to-cell transfer (SCT) method involving 60 mm Petri dishes. 21
Figure 1- 5. Schematic diagram for the surface-to-cell transfer (SCT) method involving 96-well microtiter plates in dry condition. 24
Figure 1- 6. Schematic diagram for the surface-to-cell transfer (SCT) method involving 96-well microtiter plates in wet condition. 25
Figure 2- 1. The elution of VHSV dried on a one compartment to infect EPC cells in a different compartment of the same chamber. 31
Figure 2- 2. The capacity of glass beads to transfer infectious VHSV. 34
Figure 2- 3. The capacity of fishing hooks to transfer infectious VHSV. 36
Figure 2- 4. The appearance of rust from hooks on reporter cell monolayer. 37
Figure 2- 5. The capacity of fishing lines to transfer infectious VHSV. 39
Figure 2- 6. The capacity of commercial soft drink (pop) can water bottle to transfer infectious VHSV. 41
Figure 2- 7. Survival of VHSV on plastic and glass dishes and 6-well plate inserts at room temperature dried beyond 1 day. 43
Figure 2- 8. Survival of VHSV dry on plastic dishes at different temperatures. 45
Figure 2- 9. Survival of VHSV dry on glass dishes at different temperature. 48
Figure 2- 10. The survival of various dilutions of VHSV dried at room temperature on 96-well plates. 51
Figure 2- 11. Survival of VHSV on 96-well plates in dry and wet condition at 4°C. 53
Figure 2- 12. Survival of VHSV on 96-well plates in dry and wet state at 14°C. 55
Figure 2- 13. Survival of VHSV on 96-well plates in dry and wet state at room temperature. 57
Figure 2- 14. Survival of VHSV on 96-well plates in dry and wet state at 26°C. 59
Figure 2- 15. Survival of VHSV on 96-well plates in dry and wet state at 37°C. 60
Figure 2- 16. Survival of VHSV in dry and wet conditions on 96-well plates across all temperatures. 62

Figure A- 1. Sample $TCID_{50}$ of stock VHSV showing CPE. 82

LIST OF TABLES

Table 1. Type and size of objects tested as potential fomite in OAT method. 18
Table 2. $TCID_{50/0.2\ ml}$ values derived from visual scoring and AB scoring. 27
Table 3. Agitated elution of dried infectious VHSV from different 60 mm dish surfaces and presence of infectious VHSV remaining on eluted dish 29
Table 4. The capacity of various objects to retain and transfer infectious VHSV. 33
Table 5. Survival of VHSV dry on 60 mm Petri dish surfaces at different temperatures and times. ... 46
Table A- 1. 96-well $TCID_{50}$ plate showing relative fluorescent unit (RFU) measured with AB 84
Table A- 2. Raw Alamar Blue RFU values for every wells of a sample 96-well SCT plate. 86
Table A- 3. Scores of presence of infectious virus in each well of a sample 96-well SCT plate. 87

LIST OF ABBREVIATIONS

AB	Alamar Blue
AIV	Avian Influenza Virus
AMPV	Avian Metapneumovirus
CPE	Cytopathic Effect
D-PBS	Dulbecco's Phosphate Buffered Saline
DOM	Dissolved Organic Matter
EPC	Epithelioma Papulosum Cyprinid
ET	Elution Transfer
FBS	Fetal Bovine Serum
HeV	Hendra Virus
HIV	Human Immunodeficiency Virus
L15	Leibovitz's L15 Media
LB	Low Cell Bind
NiV	Nipah Virus
OAT	Object-Associated Transfer
RFU	Relative Fluorescent Unit
RH	Relative Humidity
RT	Room Temperature
SCT	Surface-To-Cell Transfer
RTG-2	Rainbow Trout Gondal Fibroblast-Like Cell Line
VEE	Venezuelan Equine Encephalitis
VHS	Viral Haemorrhagic Septicaemia
VHSV	Viral Haemorrhagic Septicaemia Virus
VSIV	Vesicular Stomatitis Indian Virus
VSV	Vesicular Stomatitus Virus

CHAPTER 1
INTRODUCTION

1.1 Anthropogenic debris in the aquatic environment

Over the last 40 years ocean levels of man-made waste have been increasing in marine environment (Derraik, 2002). This debris not only accumulates from activities on the oceans and beaches, but also from events inland, where the resulting debris that enters streams, lakes, and rivers eventually washes to the oceans. By some estimates, nearly 80% of the marine waste comes from land sources. The nature of the debris can be quite varied, but plastic items predominate as they account for 60–80% of the waste (Gregory and Ryan, 1997). Common plastic materials are polypropylene, polyvinyl chloride, polyethylene, and polystyrene (Prutter, 1987). The plastic items float because of their low density and degrade very slowly. The result is plastic waste accumulates in gyres (swirling ocean currents), with the North Pacific gyre being best known (Moore, 2008), and on beaches, where the debris might undergo cycles of drying and wetting and possibly return to the ocean to drift to other beaches. Debris size can be used to divide marine waste into two classes: macrolitter and microlitter.

Debris items greater than 1 cm in diameter are termed macrolitter (Thiel and Gutow, 2005). This includes plastic and glass bottles, plastic bags, and styrofoam containers (Thiel and Gutow, 2005). Merchant ships, commercial fisheries, and aquaculture are significant sources of macrolitter. Merchant vessels have been estimated to dump over 600,000 plastic containers a day (Derraik, 2002). Fishing gear is abandoned or lost at sea, with estimates of approximately 135,400 tons entering oceans by this route over a year (Derraik, 2002). Aquaculture facilities lose buoys, which are used to support suspended cultures. The abundance of macrolitter in oceans can vary widely but typically ranges from 0.01 to 25 items per km^2 (Thiel and Gutow, 2005).

The majority of the plastic debris in the oceans, however, exists as small particles (< 1 cm), which is termed microlitter (Thiel and Gutow, 2005). The main source of microlitter appears to be in the manufacturing of plastic products. Tiny plastic pellets, referred to as spherules, granules, resins, cylinders or nibs are the raw material for manufacturing (Prutter, 1987). These can be spilled during loading and transport and be released in plant outfalls. Other sources of small plastic particles, usually smaller than spherules, are additives to hand cleaners, cosmetics, and airblast cleaning media (Derraik, 2002). These particles can be made of polyethylene,

polypylene and polystyrene. Additionally small plastic pellets might arise in the environment from the abrasion of larger plastic debris (Eriksson and Burton, 2003). Plastic microlittter is found in beaches, costal water, and gyres and the average mass in the oceans varies from insignificant to 0.1 kg dry weight per km^2 (Thiel and Gutow, 2005).

Both macrolitter and microlitter can be internalized by organisms. Over 180 species have been documented to ingest macrolitter. These include fish, turtles, marine mammals and birds (Derraik, 2002). The outcomes of macrolitter consumption can be drastic and include starvation and suffocation. But what is less studied is the internalization of microlitter. Ingestion has been shown in laboratory studies with amphipods, barnacles, mussels and lugworms (Browne et al., 2008; Thompson et al., 2004). In these cases the microplastic can be retained in the digestive tract, egested through defecation, or translocated through the gut epithelial lining into the tissues of the body (Browne et al., 2008). Several observations suggest vertebrates also incorporate microlitter. Medical studies have shown that plastic particles can be translocated from the gut lumen to the lymph of circulatory systems of rodents and humans (Hussain et al., 2001). Wildlife studies have detected microplastic in seal scat (Eriksson and Burton, 2003).

Organisms have been found attached to marine plastic waste and this has the potential to contribute to rafting. Several groups of plants and animals have been found on floating marine debris (Winston et al., 1997). These include red, brown, and green algae. Foraminiferans, coelenterates, polychaetes, brozoans, polychaetes, sponges, mollusks and crustaceans are among the groups of metazoans found adherent to marine plastic debris. This debris can contribute to the phenomenon of rafting, which is an important dispersal mechanism in the marine environment (Thiel and Gutow, 2005). Rafting on plastic debris might introduce species into an environment where they were previously absent or extend a species range (Winston et al., 1997). Microorganisms can also attach to plastic debris. Polystyrene spherules were found to have attached bacteria and microalgae (Carpenter and Smith, 1972). As for viruses, it is unknown whether they attach to plastic and whether attachment would enhance the movement of viruses and microorganisms in the ocean.

Recreational fishing can also produce waste in the aquatic environment, especially in freshwater lakes and streams (Radomski et al., 2006). Perhaps the types of debris that have been studied most are lead weights because they can cause lead poisoning (Scheuhammer and Norris, 1996). However, fishing lines and hooks also can be of concern. A monofilament fishing line was found in the gastrointestinal tract of manatee (Beck and Barros, 1991) and found to be harmful to coral colonies (Yoshikawa and Asoh, 2004). Anglers lose hooks both in the

environment and in fish, when their fishing line breaks. In some cases, fish with hooks die shortly after nearly being caught, whereas in other instances they live with the hooks, sometimes expelling them weeks afterwards (Doi et al, 2005; DuBois and Pieski, 2007; Tsuboi et al., 2006).

1.2 The interaction between fomites and viruses

Fomites are inanimate surfaces that are capable of supporting the survival and transmission of pathogens from one host to another. The role of inanimate surfaces acting as a mode of transmission for disease agents is of real concern in human public health and to nosocomial infections. Some human enteric viruses such as the hepatitis A virus and human rotavirus can survive dry on common household surfaces such as aluminum, paper, and latex for many days (Abad et al., 1994). Bacteriophage ΦX174, a model for poliovirus, on doorknob can consecutively contaminate 14 people (Barker et al., 2001). Fomites are also of economic concern in the animal farming industry such as the swine and poultry farming. Pseudorabies virus that infects swine can survive on concrete and steel for 4 to 7 days (Schoenbaum et al., 1991). Avian reoviruses have been shown to survive for a few days on common materials in poultry farms (Savage and Jones, 2003), and fomites are a secondary source of transmission for avian influenza in commercial poultry (Landman and Schrier, 2004). Interestingly, little attention has been given to the possible role of fomites in the aquatic environment, but in aquaculture a variety of diseases can be economically significant and understanding disease transmission is important for reducing the problem of diseases.

Viruses are the most abundant biological entity in the aquatic environment (Bergh et al., 1989). They have been estimated at densities greater than 1×10^7 particles per ml in oceans and at 10^5 to 10^9 per ml in freshwater (Sano et al., 2004). Other rather imaginative ways of expressing their abundance in nature have been presented. For instance, Suttle (2005) notes that marine viruses contain more carbon than 75 million Blue whales and that if joined end to end they would stretch further than the nearest 60 galaxies. Through their infection and lysis of microorganisms, viruses play a critical role in the cycling of nutrients (Suttles, 2007). However, all forms of life can be subject to viral infection and disease. In the marine environment, this includes not only economically important species such oysters, shrimp and fish, but also marine mammals (Lang et al., 2009). Viruses from the sea might even be able to infect terrestrial organisms (Sano et al., 2004). Whether viruses interact with anthropogenic debris in the aquatic environment is unexplored. Fish viruses would be a relevant group to study this possible

interaction because of the economic damage that they can cause and because more is probably known about them than the viruses that infect other aquatic animals.

1.3 Fish viruses

Fish virology research greatly advanced in the 1950s with the use of tissue cultures when virus was shown to be able to grow in animal tissue culture. In 1956, the blue-sac disease of rainbow trout was the first fish disease to be investigated using explants of fish tissue culture (Wolf, 1956). Two years later, the first fish cells monolayer culture was developed (Wolf, 1988). Due to the time convenience of monolayer cell culture, it eventually replaced the explant tissue culture technique in the study of fish virus. In the 1970s, researchers began focusing on the biophysical and molecular biology of fish viruses and at the same time the rhabdoviruses became the most abundant fish virus. Currently, virus causing fish diseases include both the DNA and RNA groups of viruses. Four different families of DNA viruses can infect fish. Five DNA viruses of significance are oncorhynchus masou virus and channel catfish herpesvirus belonging to the herpesviridae family, and epizootic haematopoietic necrosis virus, white sturgeon iridovirus and red sea bream iridovirus belonging to the iridoviridae family. For the RNA group, there are eleven different families of RNA viruses that are capable of infecting teleost fish. Within those 11 families are 4 families containing a total of 6 RNA viruses that have a great impact on aquaculture. These are: infectious pancreatic necrosis virus of the birnaviridae family, infectious salmon anaemia virus of the orthomyxoviridae family, nervous necrosis virus of the nodaviridae family, and spring viraemia of carp virus, infectious haematopoietic necrosis virus and viral haemorrhagic septicaemia virus of the rhabdoviridae family (Ahne, 2001).

1.4 The interaction between fomites and viruses

Viral haemorrhagic septicaemia (VHS) is a disease of salmonid fish of great economic significance in the European aquaculture industry. The viral agent that causes VHS is the viral hemorrhagic septicaemia virus (VHSV). To date, VHSV has been isolated from 37 countries spanning North America, Asia and Europe. The international database of aquatic animal diseases notes that VHSV can naturally infect about 60 different fish species. Economically important fish species that are susceptible to VHSV include Rainbow Trout, Atlantic Salmon, Cod, Herring, and turbot, with Rainbow Trout being the most susceptible (Skall et al., 2005).

The history of VHS has its beginning in Europe in the 1930s, when a new kidney swelling disease was documented in rainbow trout. However, the viral source of the disease was not

confirmed until 1962 when the virus was isolated from two outbreaks in Denmark using trout primary culture (Jensen, 1963). Initially thought to only have been confined to freshwater Rainbow Trout farms in Europe, VHSV was first isolated in European marine environment in 1979 from wild Alantic cod, Gadhus morhua. In 1988, VHSV was isolated in North America from Chinook and coho salmon; this was the first isolation of VHSV from outside of Europe (Skall et al., 2005). In 2003 and 2005, VHSV was isolated from the great lake water systems in Lake St. Clair, Michigan and Lake Ontario (Elsayed et al., 2006, Lumsden et al., 2007).

1.4.1 Physical characteristics and identification of VHSV

At the whole fish level, symptoms of VHS include behavioral changes such as the refusal to eat and lethargy. Physical signs of infected fish include a darker body with paler gills. Internal organs and muscles may show hemorrhaging. The liver undergoes necrosis resulting in a paler colour than normal. The kidneys and spleen are damaged, leading to swelling and intense reddening of those organs (Wolf, 1988).

VHS can be detected by using cell lines. Infection of cell lines, such as RTG-2 and EPC, with VHSV results in the appearance of cytopathic effect (CPE) in the cell monolayer. At the early stages of infection, CPE can be visualized as the formation of syncytia, which is the membrane fusion of many individual cells into a giant multinucleate cell (Rocha, 2004). Rounding and lysing of infected cells causes eventual breakage in some parts of the cell monolayer. At the late stages of infection, the monolayers of susceptible cells are usually completely destroyed leading to floating debris consisting of rounded cells.

At the molecular level, VHSV can be identified by three serological techniques: neutralization assays, fluorescent antibody and immunoperoxidase staining (Wolf, 1988).

1.4.2 Molecular properties and phylogenetic of VHSV

Viral haemorrhagic septicaemia virus (VHSV) is a member of the Novirhabdovirae genus belonging to the Rhadoviridae family (Bourh et al., 2005). It is an enveloped negative-stranded RNA virus with a bullet-shaped morphology. Its genome is a little over 11kb long with six genes arranged in the following order: 3'-N-P-M-G-Nv-L-5'. These six genes encode for the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonstructural protein (Nv), and a RNA-dependent RNA polymerase (L) (Schutze et al., 1999). Five of these are structural proteins with known functions and one is a nonstructural protein with no known function. While the nonstructural Nv protein showed no direct identifiable function, it has been

shown to aid in pathogenicity (Thoulouze et al., 2004). The nucleoprotein (N) binds the viral RNA genome and forms a nucleocapsid. The phosphoprotein (P) and RNA-dependent RNA polymerase (L) forms a complex to initiate viral genome replication. The matrix protein interacts with the glycoprotein, the lipid membrane and the nucleocapsid to ensure the proper formation of virions (Benmansour, 1993).

The infection cycle of VHSV begins when the transmembrane G proteins on the viral lipid envelop binds to host cell receptors, phosphatidylserine (Estepa and Coll, 1996). After binding, the G protein along with the rest of the virion is endocytosed into the cell in an endosomal vesicle. Acidification of the endosome causes the G proteins to change conformation, allowing the viral lipid envelop to fuse with the endosomal vesicle membrane. Fusion results in the release of the nucleocapsid and viral genome into the cytoplasm of host cell. Viral genome transcription and replication occur, leading to the production of new virions (Flint et al., 2000).

Phylogenetic analysis of the N and G gene showed that VHSV can be categorized into four different genotypes: I, II, III, and IV. Two of those four genotype groups are further classified into sublineages. Genotype I is classified into sublineages “a”, “b”, “c”, “d” and “e”, and genotype IV is classified into sublineages “a” and “b” (Einer-Jensen et al., 2004 and Elsayed et al., 2006). Marine VHSV belongs to all four genotype groups, whereas all freshwater VHSV belongs to genotype Ia. North American VHSV is classified into genotype group IV (Einer-Jensen et al., 2004).

1.5 Environmental factors contributing to the inactivation of microorganisms and viruses

1.5.1 Open air factor and ozone

Outdoor air, as opposed to that of indoors, contains more toxic components that adversely affect the survival of microorganisms. Druett and May (1968) compared the survival of *E. coli* in outdoor air with indoor air and found that *E. coli* died much faster in the open air. However, the actual toxic composition of outdoor air was hard to identify and changes from day to day (Druett and May, 1968), and most likely from location to location. These toxic components are hypothesized to be highly reactive chemical species that are likely created from ozone reacting with olefins (Weber, 2008).

Ozone (O_3) is a component of the atmosphere that is created from oxygen molecules by high electric discharges. It has low solubility in water and is unstable in aqueous solutions, slowly

decomposing to oxygen (Khadre, 2001). Precursors of hydroxyl radicals, which are highly reactive with hydrocarbons, can form when certain alcohols react with ozone. Ozone reacts easily with thymine and guanine in DNA, with uracil in RNA, and with the R-groups and nitrogen atoms in amino acids. Ozone can inactivate viruses, such as the rotavirus, but its effectiveness is decreased when viruses are present with organic materials from cell debris and other sources. These organic materials reduce the exposure of the virus to ozone by reacting with it (Khadre, 2001). The initial target of ozone is either the viral envelope or the protein capsid depending on whether the virus is enveloped. The destruction of the envelope and/or capsid can lead to ozone reacting with the unprotected nucleic acid genome. Ozone has been shown to effectively inactivate enveloped viruses including vesicular stomatitis Indian virus (VSIV), which is a bullet shaped virus belonging to the same rhabdoviridae family as VHSV. Electron microscopy confirmed the destruction of VSIV nucleocapsid and destruction of the lipid envelope of other viruses (Murray et al., 2008). Ozone can also inactivate virus by aggregating viral protein coats and cross-linking the protein coat with viral genome, effectively preventing viral uncoating and therefore, gene expression and replication. The above described mechanisms have been shown to be the mode of action of aqueous ozone (Khadre, 2001).

1.5.2 Ultraviolet radiation

Ultraviolet Radiation has been shown to inactivate many different types of bacteriophages and animal viruses, including the rhabdovirus vesicular stomatitus virus (VSV) (Rauth, 1965). Rauth (1965) showed that viruses with a single stranded nucleic acid genome were ten times more sensitive to UV than viruses with a double stranded nucleic acid genome. Tseng and Li (2005) showed that the UV dose for 90% inactivation of virus with double stranded nucleic acid genome was two times more than that of virus with single stranded genome. The length of the genome is also an important factor because the bigger the genome, the more targets are available for UV damage. RNA viruses are hypothesized to be generally more resistant to UV damage than DNA viruses. Thymine dimers are the most common lethal result of UV damage. Since RNA viruses do not contain thymine, they are more resistance to UV inactivation (Lytle and Sagripanti, 2005; Rauth, 1965). Viral proteins also play a role in the viral inactivation from UV light. Some viral proteins, such as those from herpes simplex and reovirus-3, are more sensitive to UV light so they increase the overall inactivation of those viruses to UV light, whereas proteins from other viruses are more resistant to UV (Rauth, 1965). Viral host cells also affect the overall survivability of virus to UV inactivation. Host cells containing DNA repair mechanisms are more effective at repairing double stranded DNA than single stranded DNA (Rauth, 1965).

1.5.3 Relative humidity and temperature

The effect of relative humidity (RH) on the survival of viruses differs markedly depending on the virus in question. At 23°C, T3 bacteriophage and infectious bovine rhinotracheitis virus survived best at 90% RH. However, Newcastle disease virus and the rhabdovirus VSV survived best at 23°C in 10% RH (Songer, 1966). Claims on the survivability of influenza at various RH are contradictory in the literature. Some groups claimed better influenza survival at low RH while others claimed at high RH (Weber and Stilianakis, 2008). Literature on other viruses suggests that different types of viruses survive best either at low or high RH and very few survive best at middle RH (Mbithi et al., 1991; Songer, 1966; Buckland and Tyrrell, 1962).

Variations in temperature can affect the survival of many different types of viruses. Venezuelan equine encephalitis (VEE), influenza, vaccinia and poliovirus were tested for survivability at temperatures ranging from 10°C to 30°C; all of these viruses survived the best at the lower temperature (Harper, 1961). Low temperatures were also shown to be favourable to the survival of airborne human coronavirus and hepatitis A virus (Ijaz et al., 1985; Mbithi et al., 1991). Frerichs et al. (2000) showed that fish nodavirus can survive and remain infective for 1 year at 15°C, 3 months at 25°C and at least 4 days at 37°C. Some viruses have also been shown to survive for a short amount of time at very high temperatures. Canine coronavirus can survive at 56°C for about 1 hour (Pratelli, 2008), and some human viruses, such as adenovirus, vaccinia and polyomavirus SV40, can survive at temperatures range of 75°C to 95°C, depending on the virus, for 1 hour (Sauerbrei and Wutzler, 2009).

1.6 Survival and/or inactivation of VHSV

A recent study showed that VHSV can survive in freshwater much better than in seawater (Hawley and Garver, 2008). In raw freshwater, VHSV can survive for 5 days or more days at 25°C, increasing to more than 25 days at 4°C. When the raw freshwater was filtered, VHSV isolates were able to survive for up to 18 times longer, depending on the isolate and the temperature. However, the survivability of those isolates was much lower in seawater, dropping to around 12 days at 4°C and about 1 day at 20°C. Filtering of the seawater did not increase the survival time but rather decreased it by a few days (Hawley and Garver, 2008). Another study showed that adding low concentrations (0.01% to 1.0%) of ovarian fluid, which is naturally secreted into the environment during spawning season, to filtered seawater increased survival of VHSV by about 1.8 to 2.4 times, respectively. However, addition of 10 ppb of crude oil to seawater, to represent oil spills in the ocean, did not affect survival of VHSV (Kocan et al., 2001).

In vivo infection of Pacific sardine with VHSV showed a major decrease in mortality, from 66.7% to 6.7%, when incubation temperature was raised from 13°C to 20°C. Commercial freezing of fish infected with VHSV resulted in about 1000 fold reduction in titer from the kidney and spleen (Arkush et al., 2006).

1.7 Factors that influence viral attachment to and survival on inanimate surfaces

Attachment of viral particles to surfaces is a two step process. The first step involves the movement of the viral particle toward the inanimate surfaces. This step is dependent on Brownian motion and the diffusion rate of the viral particle in a fluidic medium. The second step involves the actual binding of the viral particle to the inanimate surface (Gerba, 1984). One main factor affecting the attachment of the virus is the number of times the virus comes into contact with the surface before finding an attachable spot. This factor depends on the property of the virus and fluidic medium (Gerba, 1984). Hydrophobic interactions between the virus and surface are suggested to be important factors in attachment of viruses containing lipid components. Hydrophobic groups on the virus interact with hydrophobic groups on the surface. Chaotropic salts, large singly charged ions such as thiocyanate and iodide, which disrupts hydrophobic interactions, and un-ionized compounds such as Tween 80 and urea have been shown to elute virus from membrane filters and other surfaces (Gerba 1984). Antichaotropic salts, small singly charged ions such as magnesium and EDTA, which promote hydrophobic interaction, have been shown to enhance viral attachment to filters. The pH of the solution also affects the attachment of the virus, but whether high or low pH promotes or disrupts viral attachment depends on the isoelectric points of both the virus and the surface (Gerba, 1984). The isoelectric point (pI) of a particle is defined as the pH at which the particle has zero net charge; at pH above the pI, the surface or virus has a net negative charge, and below the pI, a net positive charge. The pH at which the virus and surface have opposing charge will promote attachment between them (Gerba, 1984). The presence of other organic substances in the solution can both reduce attachment of virus in general, by competitive binding, or promote the attachment of lipid-containing virus by acting as a link, binding to both the virus, if the organic substance is hydrophobic, and the surface (Gerba, 1984).

The type of surface can greatly affect the survivability of the infectious organism. Some surfaces can provide the attached viral particle with protection from inactivation. Clay has been shown to protect coliphages in freshwater and seawater, and soil and liquid containers shown to

protect poliovirus. Mechanisms of protection include: providing structural and thermal stability, and prevention from exposure to UV, proteolytic enzymes and aggregation (Gerba, 1984). However, some surfaces are detrimental to viruses. Copper surfaces have been shown to inactivate Influenza A virus much more greatly than stainless steel (Noyce et al., 2007) and copper ions in solution have been shown to reduce influenza viral titer much more than zinc ions (Horie et al., 2008). Viral inactivation of two avian respiratory viruses was shown to be greater on porous surfaces, such as cotton and wood, than on nonporous surfaces, such as steel, latex and plastic (Tiwari et al., 2006).

CHAPTER 2
MATERIALS AND METHODS

2.1 Cell culture

The cell line used to report the presence of VHSV is *Epithelioma papulosum cyprinid* (EPC) which was initially misidentified by ATCC as being from a Carp cell line (*Cyprinus carpio*) and later re-identified as a *Pimephales promelas* (Fathead Minnow) cell line. However, ATCC still maintains EPC as the designation of this particular cell line and henceforth, this report will also keep referring to the cell line as EPC. EPC cells were grown in 75 cm^2 tissue culture treated flask (BD Biosciences, San Jose, CA) at room temperature with Leibovitz's L15 media (Sigma-Aldrich, Oakville, ON) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Sigma-Aldrich, Oakville, ON). Propagation of EPC was done once every two weeks in a ratio of 1:2 using 12 mL of L15 with 10% FBS and 1% penicillin-streptomycin.

2.2 Virus propagation and identification

VHSV Type IVb, isolated from Lake Ontario, Canada (Lumsden et al., 2007), was propagated at 14°C on a confluent monolayer of EPC in 75 cm^2 flask containing fresh L-15 media supplemented with 2% FBS and 1% penicillin-streptomycin. The virus was collected from the supernatant of the flask 10 days after inoculation by centrifugation at >3000 x g followed by syringe filter through a 0.2 micrometer filter (Pall Corporation, Port Washington, NY). After filtering, virus was immediately frozen at -80°C for storage. VHSV was identified on EPC by the development of cytopathic effects (CPE), which is destruction in the EPC cell monolayer resulting in characteristic rounding of cellular debris. Viral titer was determined using the Karber method for tissue culture infectious dose ($TCID_{50}$) (Karber, 1931).

2.3 Determining tissue culture infectious dose ($TCID_{50}$) of VHSV using the Karber method

EPC cells were seeded into wells of a 96-well tissue culture treated microtiter plate (BD Biosciences, San Jose, CA) at about 50 000 cells/well. Cells were allowed to attach and form a monolayer overnight at room temperature. The next day, an unknown stock concentration of filtered VHSV was serially diluted 10-fold from stock to 10^{-9} in L-15 with 2% FBS and 1%

penicillin-streptomycin. Old media from the 96-well plate containing a monolayer of EPC cells was removed by inverting the plate and blotting it on a stack of paper towels. In the first column of the 96-well plate, 200 µl of L15 with 2% FBS and 1% penicillin-streptomycin was added per well for 6 wells. In the next column, 200 µl of the 10^{-1} dilution of VHSV was added to each well for a total of 6 wells. This process was repeated for adjacent columns until the very last dilution (10^{-9}) of VHSV was added to the plate. The plate was then incubated at 14°C for 7 to 10 days to allow development of CPE. Figure 1-1 shows a graphical representation of the setup of $TCID_{50}$ of a 96-well plate described above. Each well was scored for presence of virus by visually checking for appearance of CPE. $TCID_{50}$ was calculated using the Karber method (a sample calculation can be seen in Appendix 1).

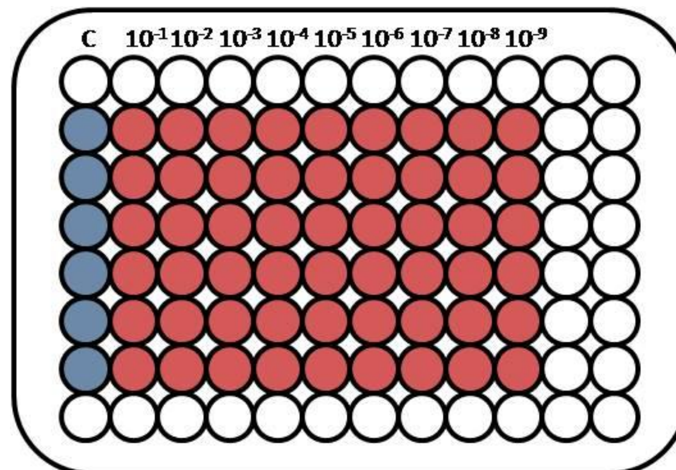

Figure 1- 1. TCID50 setup on a 96-well microtiter plate.

Row 1 (blue) of the 96-well plate has 6 control wells containing 200 µl of L15 with 2% FBS, labeled as “C” on the image. Row 2 (red) has 6 wells exposed to 200 µl of 10^{-1} dilution of VHSV in L15 with 2% FBS. Every adjacent row is exposed to a 10 fold higher dilution of VHSV up to 10^{-9}.

2.4 Scoring $TCID_{50}$ plates with Alamar Blue (AB) assay

Four 96-well plates were seeded with 50,000 EPC cells/well in 100 µL of L15 with 10% FBS and 1% penicillin-streptomycin and left overnight to form a monolayer (the setup is the same as in the section 2.3). Four separate $TCID_{50}$ assays of the same stock VHSV were performed on those plates. The plates were incubated at 14°C for 7 to 10 days to allow for the development of CPE. After incubation, the four plates were visually examined and each exposed well was scored as positive or negative for the presence of CPE. Alamar Blue (AB) assay was subsequently performed on those plates to measure reporter cell viability using the following protocol. Old media from each plate was removed by inverting the plate and blotting the media onto a paper towel. The wells were then washed with 100 µL of L15/ex. AB dye (5% v/v diluted in L15/ex) was then added to each well with a total volume of 100 µL per well. The cells were incubated with AB for about 1 hour and subsequently measured with the SpectraMax GEMINI XS plate reader at an excitation wavelength of 530 nm and emission wavelength of 595 nm. The resulting output of the assay is given in relative fluorescent unit (RFU). The RFUs of the control wells in each plate were averaged. The RFU of each exposed well was compared to the average RFU values of the control wells and if the RFU value of an exposed well is lower than half of the control average (the cut-off criteria), the well was scored positive for infectious virus. The number of wells with CPE scored visually and the number of wells with infectious virus scored by AB were used to calculate the $TCID_{50}$ values of the four plates and compared to determine if there are differences in titer between visual and AB scoring. See Appendix 2 for a sample scoring of CPE using AB.

2.5 Detection of VHSV attachment and survival dried on various surfaces using the agitated elution transfer (ET) method

In this method, VHSV was seeded onto dishes with different surfaces and then dried at room temperature. The dried VHSV was then eluted from the dishes using different elution solutions and the amount of VHSV eluted was quantified using the $TCID_{50}$ method. A detailed account of the agitated ET method is as follows. Three different 60 mm dish surfaces containing 6 mL of L15 with 2% FBS were seeded with 400 µl of stock VHSV($10^{8.8}$ $TCID_{50/200\ ul}$): low cell bind (Nunc, Tokyo, Japan) , conventional tissue culture treated (Corning, New York, USA), and soda lime glass dishes (VWR International, Mississauga, ON). The dishes were incubated at 14 °C for 24 hours, to allow virus to potentially bind to the surfaces, and then washed with 6 mL of D-PBS (Sigma-Aldrich, Oakville, ON). They were left to dry at room temperature for 6 days. The dried

dishes were eluted with different elution solutions: L-15 with 2 % FBS, L-15 with 10 % FBS, tissue culture grade water, seawater, and reference freshwater. The elution protocol begins with initially covering the dried surfaces with 1 mL of individual elution solutions followed by incubation for 5 minutes at room temperature. Afterwards, the 1 mL elution solution was pipette mixed on the surface 5 times with a P1000 Eppendorf pipette in an attempt to dislodge any bound VHSV on the surface. The mixed elution solution was transferred to a 1.5 mL microcentrifuge tube and then frozen at -80 ^{o}C. The frozen elution solutions were later thawed and titrated using the Alamar Blue $TCID_{50}$ method at a later convenient time. The schematic diagram of the agitate ET method can be seen in Figure 1-2.

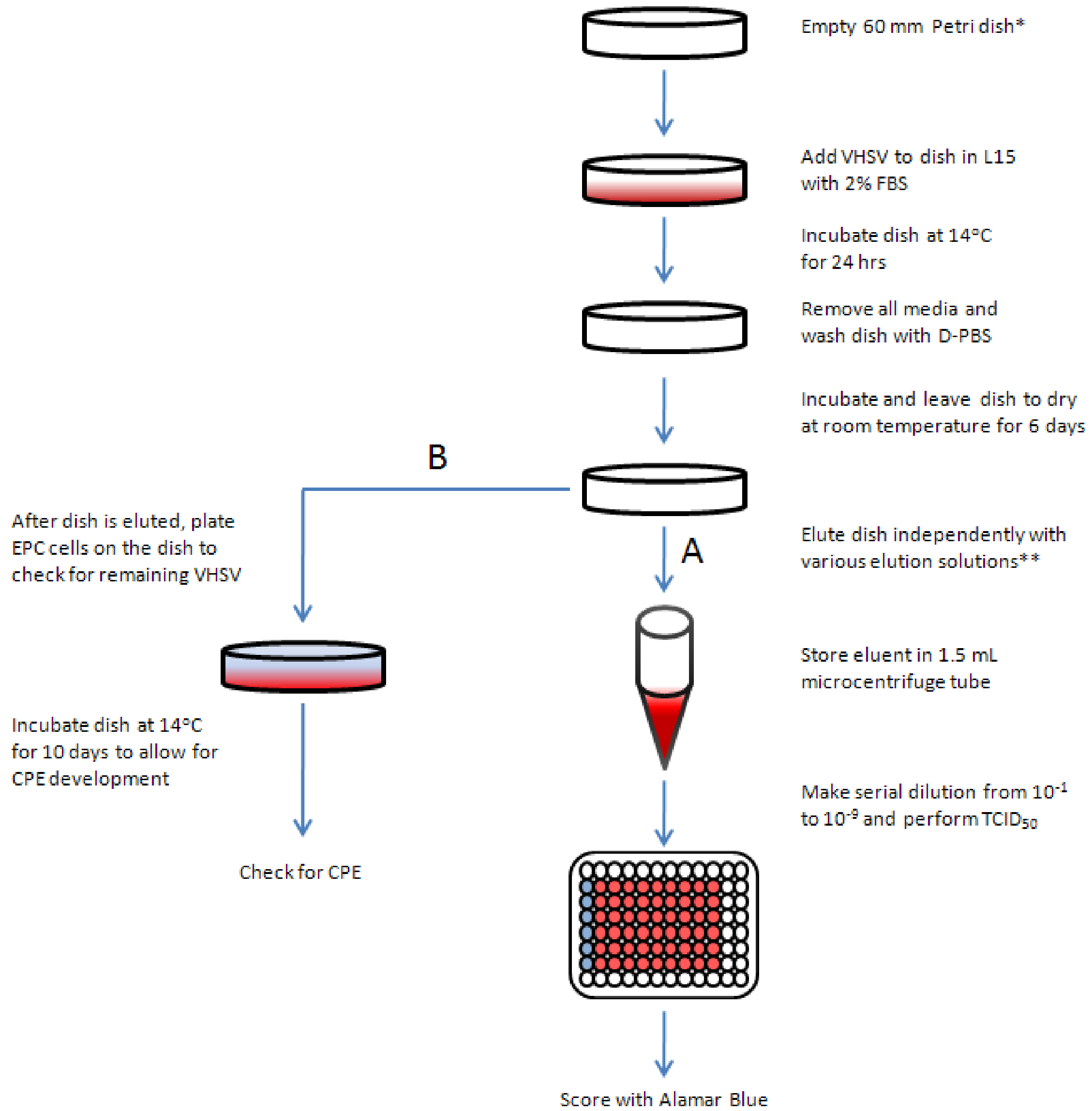

Figure 1- 2. Schematic diagram for the agitated elution transfer (ET) method.

Detailed procedure of this method is described in Section 2.5. Step A in the diagram occurs before step B.
*Three different types of dishes were used: low cell bind, conventional tissue culture treated polystyrene, and soda lime glass dish.
**Five different types of elution solutions were used: tissue culture water, L15 with 2% FBS, L15 with 10% FBS, reference freshwater, and seawater

2.6 Detection of VHSV attachment and survival dried on PET track-etched membrane cell culture insert using the static elution method

Two-compartment chambers are created by placing a 6-well cell culture insert into a well of a 6-well plate. The insert sits inside the well on the plate leaving a distance of about 1 mm between the bottom of the insert and bottom of the well. The base of the insert is a PET track-etched membrane containing numerous randomly displaced 3.0 µm pores. The pores serve as the only points of entry between the two compartments.

There are two setups for the two-compartment chamber methods. The first setup involves drying VHSV in the top insert compartment on the PET membrane then moving the insert into a well of a 6-well plate containing EPC cells. In this setup, the virus is dried on the top compartment while reporter cells are in the bottom compartment. The detailed protocol for the first setup of the two-compartment chamber method is as follows. Three 6-well cell culture inserts (BD Biosciences, San Jose, CA) were added to 3 wells of a 6-well plate (BD Biosciences, San Jose, CA). To one of the 3 inserts, 6.4 mL of L15 with 2% FBS was added; this is the control. To the remaining two of the 3 inserts, 6 mL of L15 with 2% FBS along with 400 µl of stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) were added. The 6-well plate containing the 3 inserts was incubated at 14°C for 24 hours. After 24 hours, the inserts were washed with 6 mL of D-PBS and then left to dry at room temperature for 6 days. After this, the 3 inserts were removed from the wells of the original 6-well plate and moved to a new 6-well plate containing 2.6 million EPC cells already pre-plated. The new plate containing the insert was incubated at 14°C for 7 to 10 days to monitor for CPE.

The second setup involves drying VHSV in the wells of 6-well plate and then placing into those wells inserts containing EPC reporter cells. In this method, the virus is dried in the bottom compartment and reporter cells are in the top compartment. The protocol for this setup is similar to the first setup except that the location of the virus and the reporter cells are reversed.

2.7 Detection of VHSV attachment and survival dried on various potential fomites using the object-associated transfer (OAT) method

In this method, VHSV was exposed to the surface or object in study. After the object was kept dry or wet with VHSV for various times up to 10 days, the object is moved from the location of incubation into a tissue culture vessel that already has a monolayer of EPC cells to monitor for

CPE development. A more detailed account of the OAT method is as follows. Initially, objects of various sizes and dimensions (Table 1) were placed separately in a 100 mm Petri dish containing 10 mL of L15 with 2% FBS and 400 μL of stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) and incubated at 14 ^{o}C for 24 hours. Media from the dishes containing the object and VHSV was then removed and the dish was washed with 10 mL of D-PBS. In the dry condition, the dish containing the object was incubated at room temperature after washing and removing the wash solution. For the wet condition, after washing and the removal of wash solution, 10 mL of L15 with 2% FBS was added to the dish. The dish was incubated at room temperature. For both conditions, the incubation times were for 1, 6, and 10 days. At each of those time points, the dried or wet object was removed from the dish and was transferred to a 60 mm reporter dish containing fresh L15 with 2% FBS and a confluent monolayer of EPC pre-seeded the night before. The reporter dish was incubated at 14°C for 10 days to monitor CPE development. The schematic diagram of the OAT method with glass beads in dry condition can be seen in Figure 1-3.

Table 1. Type and size of objects tested as potential fomite in OAT method.

Object	Dimension
Soda Lime Glass Beads	4 mm
Danielson Baitholder Hooks, Fishing Hook	Size 8
Mustad Beak Hook, Fishing Hook	Size 8
Northern Sport Northern Braid, Fishing Line	50.8 mm (2 inches)
Spiderwire EZ Super Mono, Fishing Line	50.8 mm (2 inches)
mini Sprite pop can pieces	10 mm x 10 mm
Nestle PureLife 500 ml water bottle pieces	10 mm x 10 mm

Preparation of object: The glass beads and the two fishing hooks were removed from their packaging and autoclave sterilized before the beginning of the experiment. The fishing lines were cut from the longer cords using scissor to a length of about 50.8 mm (2 inches) per line and sterilized using immediate immersion in 70% ethanol and overnight exposure to UV. The mini Sprite pop can and Nestle water bottle was cut to square pieces (10 mm x 10 mm) and sterilized using immediate immersion in 70% ethanol and overnight exposure to UV.

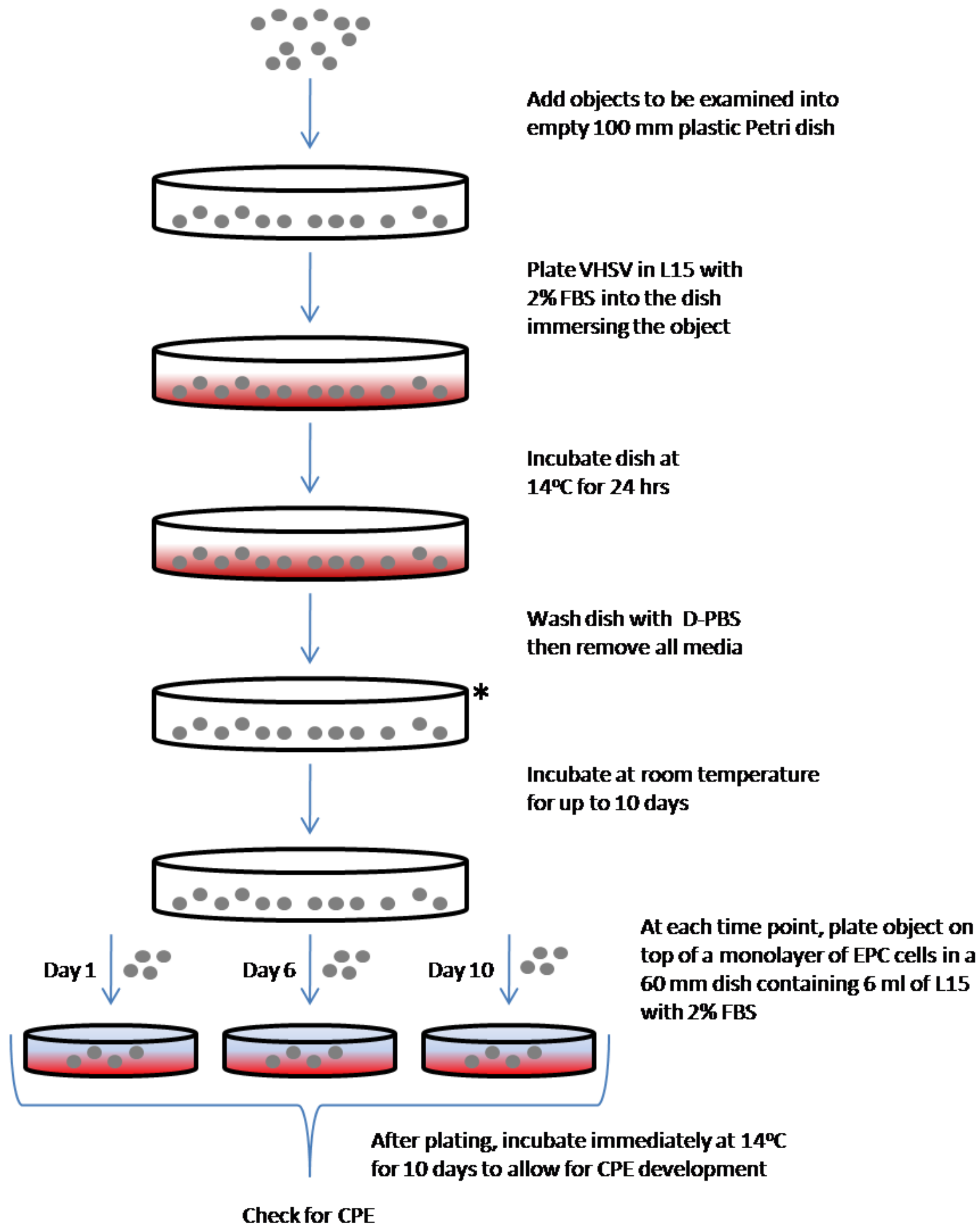

Figure 1- 3. Schematic diagram for the object-associated transfer (OAT) method involving glass beads in dry condition.

This is a schematic diagram for the OAT method involving glass beads. Detailed procedure of this method is described in Section 2.7 and is the same for other objects. The objects tested are listed in Table 1.
* For the wet condition, 10 mL of L15 with 2% FBS is added to the dish at this step.

2.8 Detection of VHSV attachment and survival in dry and wet conditions at different temperatures on various 60 mm dishes using the surface-to-cell transfer (SCT) method

The third method is termed surface-to-cell transfer (SCT) of virus because both the cells and the culture medium likely contribute to the elution of the virus from the surface and the cells report the presence of the virus by developing CPE. This method was done with plastic tissue culture surface and soda lime glass surfaces. The plastic vessels were either the 60 mm tissue culture treated polystyrene Petri dishes or PET tract-etched cell culture insert. The glass vessels were 60 mm Petri dishes. In this method the EPC cells were plated directly onto these surfaces after they have been incubated with virus in a dry or wet environment at various temperatures and for up to 15 days. The EPC cells were in L-15 with 2 % FBS and added to give a complete monolayer on the surfaces that had been previously exposed to virus. The presence of virus on the surface is indicated by the development of CPE.

A more detailed account of the SCT method using 60 mm polystyrene and glass dishes done in the dry condition is as follows. The dishes were seeded with 400 µL of stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) and 6 mL of L15 with 2% FBS. The dishes were incubated at 14°C for 24 hours to allow VHSV to settle and to potentially attach to the surface of the dishes. After the initial incubation, media from the dishes was removed and the dishes were washed with 6 mL of D-PBS. Afterward, the dishes were then incubated to dry at either 4°C, 14°C, room temperature, 26°C or 37°C for 1, 6, 10, or 15 days. At each of these time points, EPC cells in L-15 with 2% FBS were plated on the dishes and then the dishes with cells were incubated at 14°C for 10 days to monitor CPE development. The schematic diagram of the SCT method involving 60 mm dishes can be seen in Figure 1-4. For the cell culture insert, the protocol is the same except that it was only dried at room temperature and for exactly 6 days.

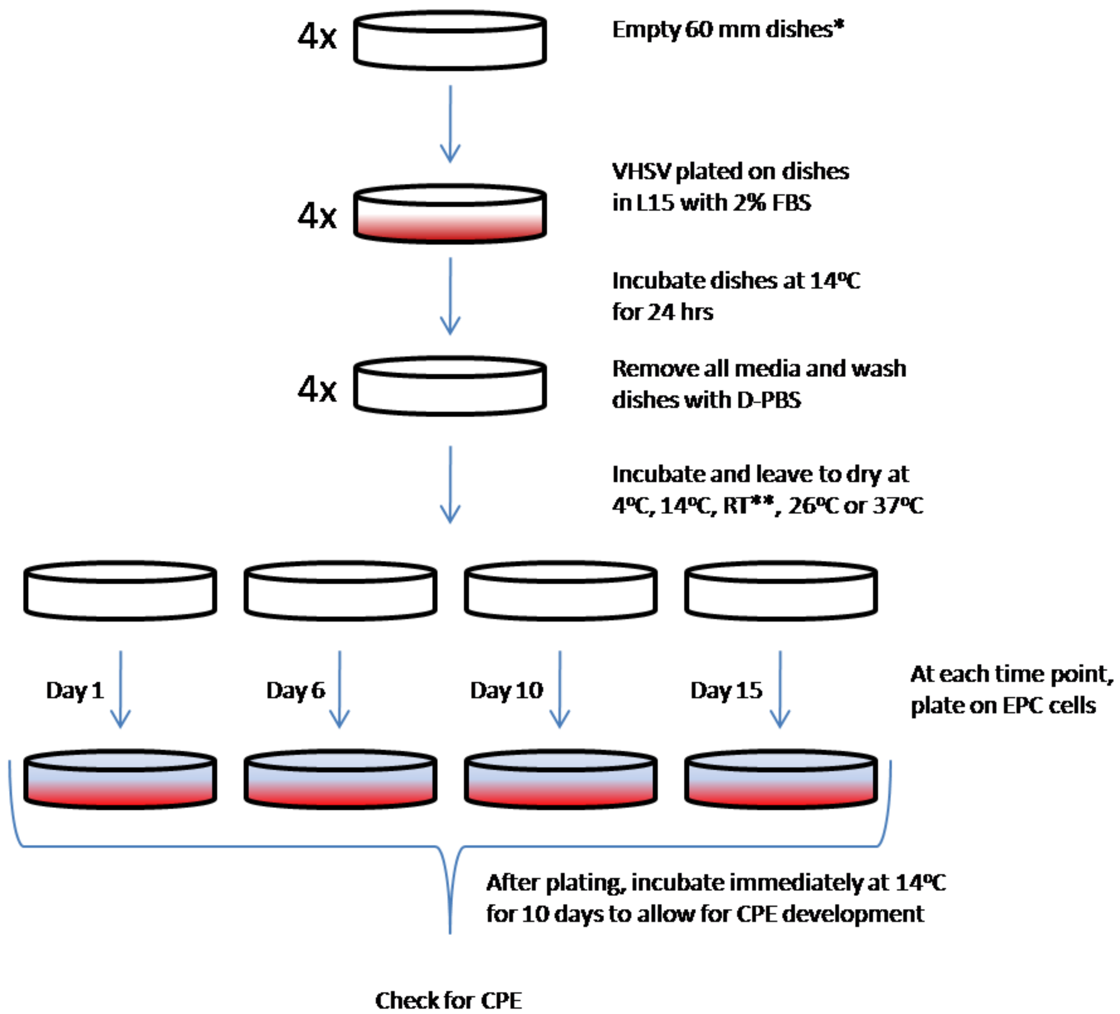

Figure 1- 4. Schematic diagram for the surface-to-cell transfer (SCT) method involving 60 mm Petri dishes.

Detailed procedure of this method is described in Section 2.8.
*Two different types of dishes were used: conventional tissue culture treated polystyrene and soda lime glass dish.
**RT stands for room temperature.

2.9 Relative quantification of VHSV attachment and survival in dry and wet conditions at various temperatures on 96-well microtiter plates using the surface-to-cell transfer (SCT) method

A variation of SCT method was developed to get a relative quantitative idea of virus survival on polystyrene surface at various temperatures. For this method, VHSV was seeded on 96-well microtiter plates instead of 60 mm plastic dishes. The premise of this relative quantitative method is based on the hypothesis that if the inactivation of VHSV by drying at various temperatures is not uniform throughout the surface of the plate, then some wells on the plate could have all VHSV inactivate while other wells do not. Therefore, by counting all the wells on the plate with and without infectious virus, the relative survival of VHSV dried at different temperature can be compared. The survival of VHSV on 96-well plates incubated wet in L15 with 2% FBS at various temperatures can also be compared.

A detailed account of the 96-well plate SCT method is as follows. Each 96-well plate is unevenly divided into the control section and the VHSV exposed section; column 1 and 2 (a total of 16 wells) of the 96-well plate represent the control section and column 3 to 12 (a total of 80 wells) represent the VHSV exposed section. Stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) was diluted to 10^{-2} in L15 containing 2% FBS. To each well in column 3-12 of each plate (80 exposed well), 100 µl of the diluted VHSV was added. To each well in column 1-2 of each plate (16 control wells), 100 µl of L15 with 2%FBS (no virus) was added. All plates were then incubated at 14°C for 24 hours to allow for the virus to settle and potentially bind to the bottom or wall of the wells. After 24 hours, old media from the plates were removed and the wells in each plate were washed with 100 µl of Dulbecco's PBS (D-PBS) (Sigma-Aldrich, Oakville, ON). Control wells and exposed wells on the same plate were washed separately to prevent cross contamination. For the plates to be incubated dry the wash solution was removed and the plates were immediately incubated at either 4°C, 14°C, room temperature, 26°C or 37°C for 1, 6, 10, or 15 days. At each of these time points, EPC cells in L-15 with 2% FBS were plated into the wells and the plates were incubated at 14°C for 10 days to monitor viral survival. For the wet condition, after washing, 100 µl of fresh L15 with 2% FBS was added to each well. The plates were then sealed with parafilm and incubated at either 4°C, 14°C, room temperature, 26°C or 37°C for 1, 6, 10, or 15 days. At each of these time points, old media was removed from the wells and EPC cells in L-15 with 2% FBS were plated into the wells. The plates with cells were incubated at 14°C for 10 days to monitor viral survival. After 10 days, Alamar Blue assay was used to score each control and exposed well on the plates

as either positive or negative for presence of infectious virus. The total number of wells scored positive for infectious virus for each plate was tallied. See Appendix 3 for a sample scoring of the 96-well plate SCT method using Alamar Blue. The schematic diagram of the 96-well plate SCT method can be seen in Figure 1-5 for dry condition and Figure 1-6 for wet condition.

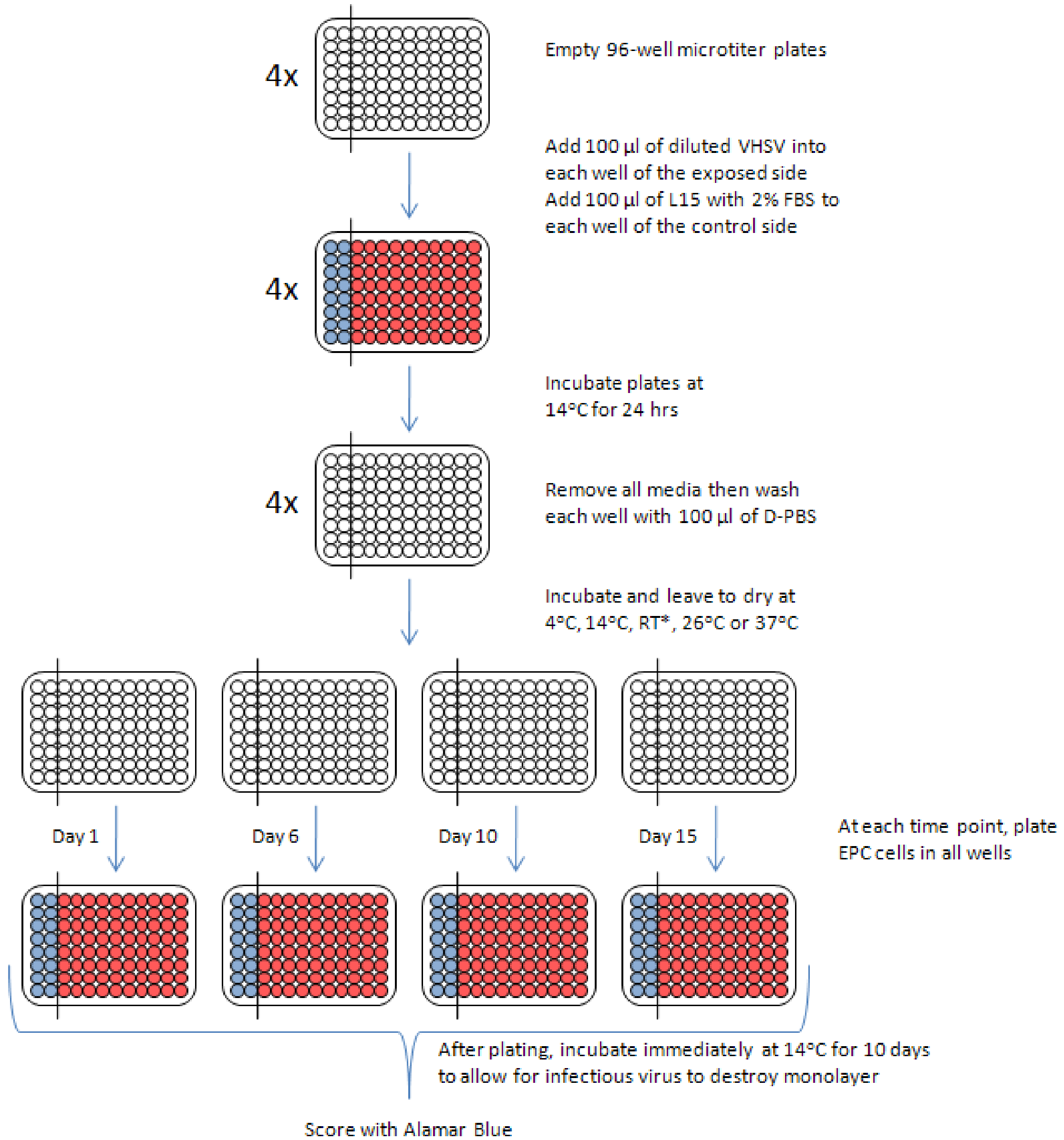

Figure 1- 5. Schematic diagram for the surface-to-cell transfer (SCT) method involving 96-well microtiter plates in dry condition.

Detailed procedure of this method is described in Section 2.9.
* RT stands for room temperature

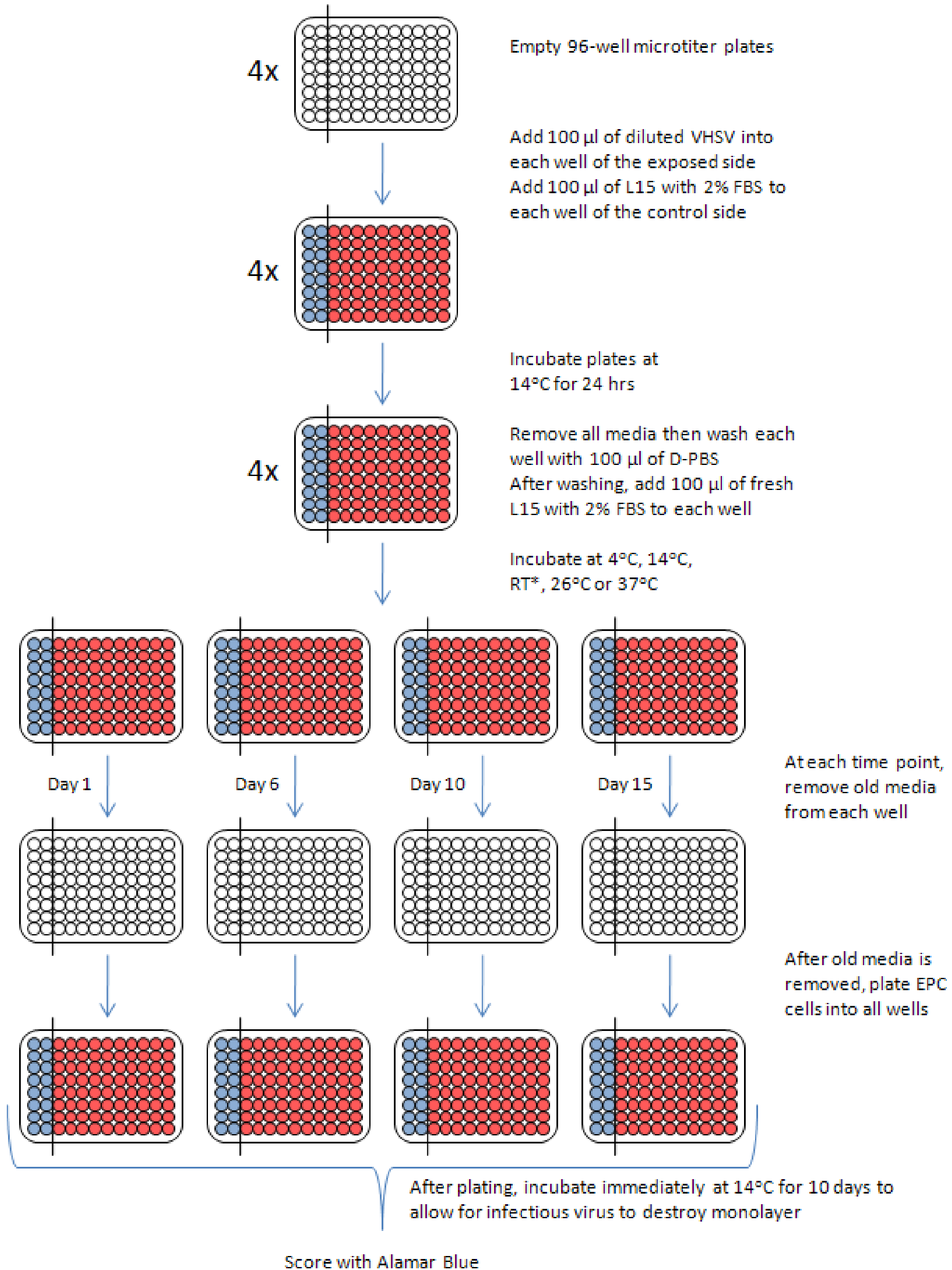

Figure 1- 6. Schematic diagram for the surface-to-cell transfer (SCT) method involving 96-well microtiter plates in wet condition.

Detailed procedure of this method is described in Section 2.9.
* RT stands for room temperature

CHAPTER 3
RESULTS

Conventionally, when performing a $TCID_{50}$, wells of a 96-well plate are scored for presence or absence of VHSV by visually examining for characteristics of CPE under a microscope. However, instead of visually checking for CPE, an indirect scoring method is proposed using the AB assay. This is an indirect method in that it does not actually confirm the presence of CPE but instead it measures the relative viability of EPC reporter cells in VHSV exposed wells and in control wells in terms of relative fluorescent units (RFU). If the RFU of individual exposed well is lower than half of the average RFU of control wells (the cut-off criteria) then that particular well is scored positive for containing infectious VHSV.

3.1 Comparison of $TCID_{50}$ values derived from visual scoring vs. AB scoring of $TCID_{50}$ plates

Viral titer calculation ($TCID_{50}$) of stock VHSV was performed on four replicate 96-well microtiter plates with a total of 6 wells exposed per dilution of VHSV per plate. The wells of each plate were scored visually for CPE before being subjected to AB cell viability measurement. Both scores were used to separately calculate the $TCID_{50}$ of each plate and the results were compared. Table 2 shows that for each of the four $TCID_{50}$ plates, the resulting titer calculated from both the visual and the AB methods are the same. This confirms that the AB method can be used to indirectly score for presence of infectious VHSV.

Table 2. $TCID_{50/0.2\ ml}$ values derived from visual scoring and AB scoring.

Plates	$TCID_{50/0.2\ ml}$	
	Visual Scoring	**AB Scoring**
1	$10^{8.83}$	$10^{8.83}$
2	$10^{9.0}$	$10^{9.0}$
3	$10^{8.67}$	$10^{8.67}$
4	$10^{8.83}$	$10^{8.83}$

The $TCID_{50}$ plates were prepared as described in Section 2.4 of the Materials and Methods section. AB scoring is done as described in Appendix 2 and the titer values are calculated as described in Appendix 1 using the Karber formula for $TCID_{50}$.

3.2 Examining VHSV attachment and survival dried on various 60 mm dish surfaces and PET track-etched membrane using the elution transfer (ET) method

The elution of VHSV from dried surfaces was examined using two different elution transfer techniques. The first method is referred to as the agitated elution of VHSV, and the second method is referred to as the static elution and was tried in order to elute without any pipetting or mechanical action.

3.2.1 Agitated elution

Briefly, for the agitated elution method, VHSV was dried on low cell bind, conventional tissue culture treated, and soda lime glass dishes for 6 days at room temperature. After drying, VHSV was eluted with five different elution solutions: L-15 with 2% FBS, L-15 with 10% FBS, tissue culture grade water, seawater, and reference freshwater, to dislodge and recover any potentially infectious viruses. Dislodgement was enhanced by pipetting these solutions up and down five times with a P1000 pipette, which is the mechanical action of this method.

Infectious VHSV was recovered in eluent after 6 days of drying from low cell bind dishes using all elution solutions listed in Table 3 except for tissue culture water but was only recovered from conventional tissue culture dishes when L15 with either 2% or 10% FBS was used to elute (Table 3). Although infectious VHSV was eluted, the amount was unable to completely infect all the wells in the first dilution column when $TCID_{50}$ was performed. Therefore, the amount of VHSV recovered was too small and could not be used to calculate $TCID_{50}$ values. Infectious VHSV was not recoverable in eluent from soda lime glass dishes regardless of the elution solution used (Table 3).

When EPC reporter cells were added onto the all the dishes after elution, only low cell bind and conventional tissue culture dishes that was eluted with L15 with 2% FBS and L15 with 10% FBS contained infectious VHSV that remained on the surface of the dishes (Table 3). All glass dishes and all other dish types that were not eluted with L15 with either 2% FBS or 10% FBS did not retain any infectious VHSV on their surfaces (Table 3).

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Table 3. Agitated elution of dried infectious VHSV from different 60 mm dish surfaces and presence of infectious VHSV remaining on eluted dish.

Elution Solutions	Petri dish surfaces and presence virus in eluent and in eluted dishes					
	Low cell bind dishes		Conventional tissue culture dishes		Soda lime glass dishes	
	Eluent	Dishes	Eluent	Dishes	Eluent	Dishes
Tissue culture water	-	-	-	-	-	-
L15 with 2% FBS	+	+	+	+	-	-
L15 with 10% FBS	+	+	+	+	-	-
Simulated freshwater	+	-	-	-	-	-
Seawater	+	-	-	-	-	-

All negative signs indicate that infectious VHSV was not present in eluent or dish. All positive signs indicate that infectious VHSV was present in eluent and dishes. However, the recovered amount in the eluent was too little and cannot be used to calculate the $TCID_{50}$.

3.2.2 Static elution

In the first setup, when VHSV was dried on the top insert compartment and reporter cells were added to the bottom well compartment, the virus was eluted by L15 with 2% FBS from the top insert compartment, entered through the pores of the insert and infected reporter cells in the bottom well compartment. Figure 2-1, Panel B shows a monolayer of reporter cells in the bottom well compartment destroyed by VHSV eluted from the top insert compartment whereas the monolayer of reporter cells in the control (Panel A) is intact. In the second setup, when VHSV was dried on the bottom well compartment and reporter cells were added to the top insert compartment, the virus was eluted from the bottom, entered through the pore in the insert membrane to infect and destroy the reporter cell monolayer (Panel D). However, when no virus was added to the bottom compartment (Panel C), the control for the experiment in Panel D, the monolayer of reporter cells remains intact on the insert membrane.

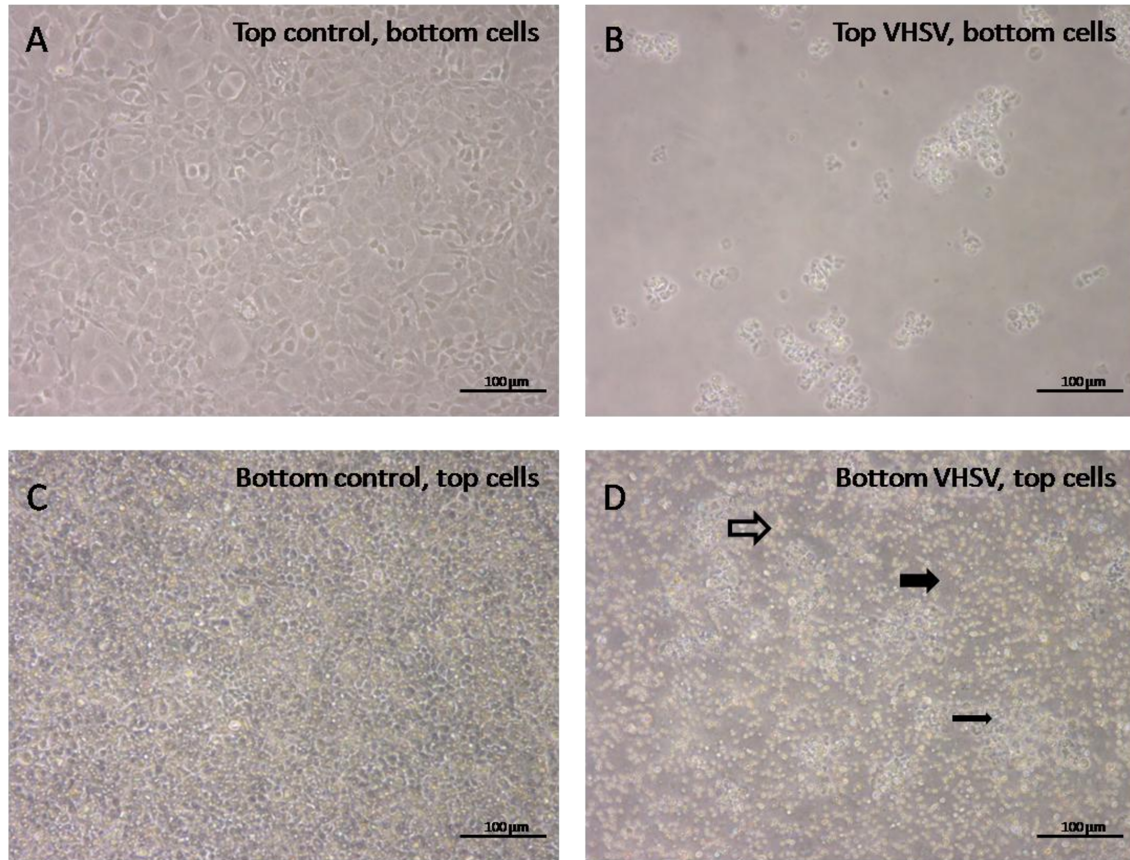

Figure 2- 1. The elution of VHSV dried on a one compartment to infect EPC cells in a different compartment of the same chamber.

6 ml of 2 % FBS/L-15 without VHSV were added to the top insert compartment (A) and the bottom well compartment (B) on separate plates. These are the controls. 6 ml of 2 % FBS/L-15 with 400 µl of VHSV at $10^{8.8}$ $TCID_{50/200\ ul}$ were added to the top insert (C) in the same plate as in (A) and the bottom well compartment (D) in the same plate as in (B). The 6-wells plates containing the inserts were rinsed with D-PBS after incubation at 14°C for 24 hours. The plates were then dried at room temperature for 6 days. After drying, the inserts in the 6-wells plates (A and B) were moved into a new 6-wells plate containing EPC reporter cells in 3 ml of 2 % FBS/L-15. After the insert was transferred, 3 ml of 2 % FBS/L-15 was added to the insert compartment. For the 6-wells plate that started with control media and VHSV in the bottom wells (C and D), 3 ml of 2 % FBS/L-15 were added to each well and new inserts was placed into the wells after the drying period. EPC cells were then added into the new inserts in 3 ml of 2 % FBS/L-15. The media in the top insert compartment and bottom well compartment mixed through the pores in the insert membrane. The plates were incubated at 14°C for 7 to 10 days to allow for CPE development. In the controls, a monolayer of cells covers the surface of the bottom well compartment (A) and top insert compartment (B). In B, the monolayer of cells in the bottom well compartment is destroyed by VHSV that was dried on the top insert compartment. In D, the monolayer of cells in the top insert compartment is destroyed by VHSV that was dried on the bottom well compartment. The small solid arrow indicates floating cell debris. The large solid arrow indicates an empty area on the insert surface with no cell or pores. The large hollow arrow indicates a group of pores. These pores are scattered throughout the surface. The images were photographed at 200 X with scale bars showing 100 µm.

3.3 Examining VHSV attachment and survival on various objects using the object-associated transfer method

3.3.1 Soda lime glass beads

When soda lime glass beads were left to dry for one day after exposure to VHSV and subsequently transferred to reporter cells, VHSV was transferred along with the beads resulting in destruction of the reporter cells monolayer (Figure 2-2, Panel A). When the glass beads were left to dry for 6 days and beyond, infectious VHSV was not transferred along with the beads because the reporter cells monolayer did not show CPE and remained intact (Table 4). However, all glass beads that were incubated wet for up to 10 days after VHSV exposure were able to transfer VHSV into reporter cells monolayer (Table 4). Panel B shows the reporter cell monolayer destroyed after coming into contact with viral exposed glass beads that were incubated wet for 10 days.

Table 4. The capacity of various objects to retain and transfer infectious VHSV.

Objects	State after rinsing the object and ending virus exposure	Presence of CPE Time the object was left to incubate at room temperature (days)		
		1	6	10
Soda Lime Glass Beads	Dry	+	-	-
	Wet	+	+	+
Danielson Baitholder Hooks, Fishing Hook	Dry	-	-	-
	Wet	+	-	-
Mustad Beak Hook, Fishing Hook	Dry	-	-	-
	Wet	+	+	-
Northern Sport Northern Braid, Fishing Line	Dry	+	-	-
	Wet	+	+	+
Spiderwire EZ Super Mono, Fishing Line	Dry	+/-*	-	-
	Wet	+	+	+
mini Sprite pop can pieces	Dry	+	-	-
	Wet	+	+	+
Nestle PureLife 500 ml water bottle pieces	Dry	+	+/-*	-
	Wet	+	+	+

All objects were exposed to the same amount of VHSV and incubated under similar dry and wet conditions at room temperature. For every object type, except for the glass beads, one object was transfer to individual reporter cell monolayer at each time point and incubation condition. For glass beads, four beads were transferred to individual reporter cell monolayer instead of one.
*Inconsistent results with some trial showing positive results while other showed negative

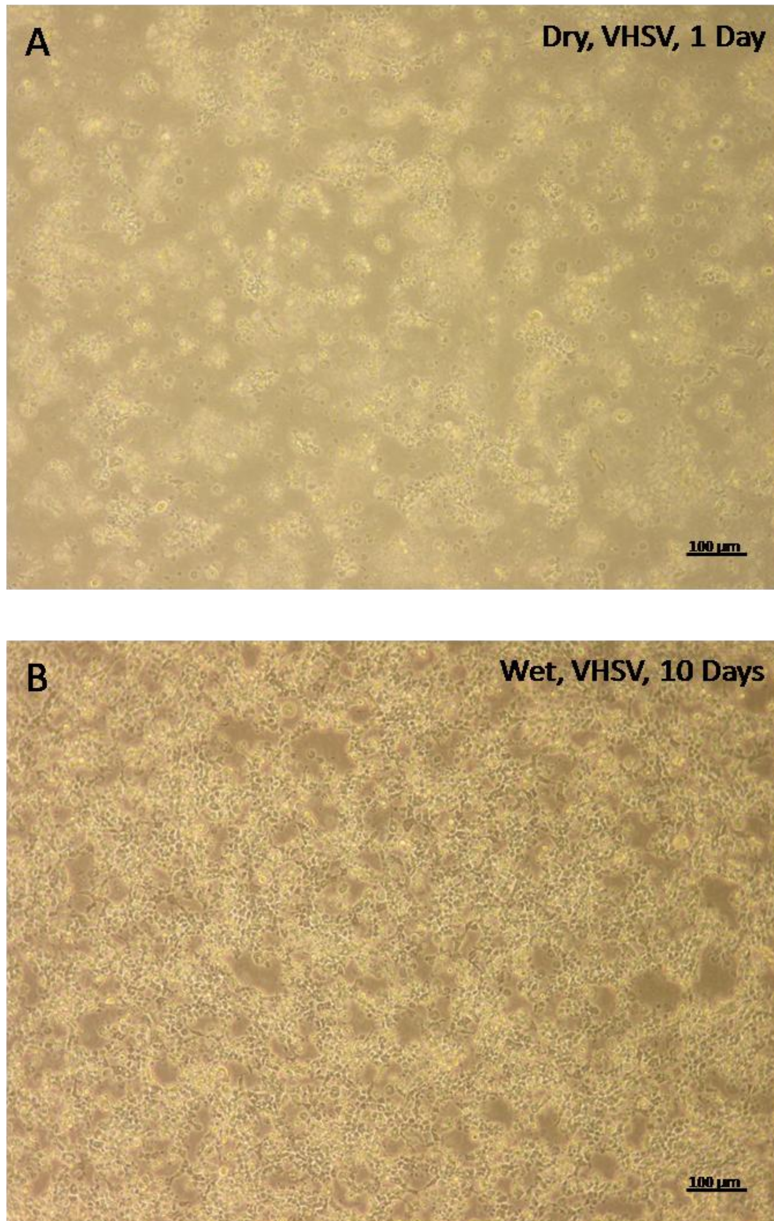

Figure 2- 2. The capacity of glass beads to transfer infectious VHSV.

Two sets of glass beads (A and B) were inoculated with 400 µl of stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) in 10 ml of 2% FBS/L15 for 24 hours at 14°C. Both sets were then rinsed with D-PBS. Set A was left to dry at room temperature for 1 day, while 10 ml of fresh 2% FBS/L15 was added to the beads in set B which was left at room temperature for 10 days. After each incubation time, 4 beads from the dry set (A) and the wet set (B) were separately added to a dish containing a monolayer of EPC cells. All cultures were then incubated at 14°C for 7 to 10 days prior to being photographed at 100 X with a camera mounted on an inverted phase-contrast microscope. In A, the monolayer shows a small degree of destruction resulting in cell-free regions of plastic surface in the monolayer (arrow), which is the CPE. In B, monolayer was completely destroyed leaving floating cellular debris and rounded cells to represent CPE. In the no virus controls for both A and B, the EPC monolayers were completely intact (data not shown). The scale bar shows 100 µm.

3.3.2 Fishing Hooks

When two different types of fishing hooks were left to dry after exposure to VHSV, neither types were able to transfer infectious VHSV into reporter cell monolayer (Table 4). Figure 2-3, Panels A and C show reporter cell monolayers completely intact after coming into contact with VHSV exposed Danielson and Mustad brands fishing hooks, respectively. When the two types of hooks were incubated in wet condition after VHSV exposure, the Danielson brand hook was able to transfer infectious VHSV after 1 day of incubation (Panel B) but not by the 6th day. The Mustad brand hooks were able to transfer infectious VHSV after 6 days of incubation (Panel D) but not by the 10th day. One important observation regarding the fishing hook experiment is the development of rust from the hooks as they were incubated dry and wet for 10 days after viral exposure. The rust that formed around the hooks was also transferred into the reporter cell monolayer along with the hooks. Further rusting of the hooks also occurred during the time that the hooks were incubated with the reporter cells. The rust produced by the hooks was toxic to the reporter cells in a concentration gradient dependent manner as cells closest to the hooks died while those that are farther away did not. The rust from the hooks was never enough to completely destroy the reporter cell monolayer even after 10 days of incubation with the monolayer. This fact differentiates death of reporter cells from hooks with death of reporter cells from infectious VHSV. Death from VHSV is usually total in that the monolayer is completely destroyed by the 10th day or at random spots throughout the monolayer that is not concentration gradient dependent. Figure 2-4 shows reporter cells death by rust from Mustad brand hooks that were not exposed to VHSV (Panel A); notice the short concentration gradient dependent of cell death as cells far enough away from the hook did not die. Panel B shows an image of a completely intact area of the same monolayer as in Panel A except taken far away from the hook. Panel C shows an image of reporter cells death, by Mustad brand hooks exposed to VHSV, taken near the hooks and Panel D shows the same monolayer destroyed when the image was taken far from the hook such that the hook is not in the field of view. Although only pictures of Mustad brand hooks were shown, rust was confirmed to be present from both types of hooks.

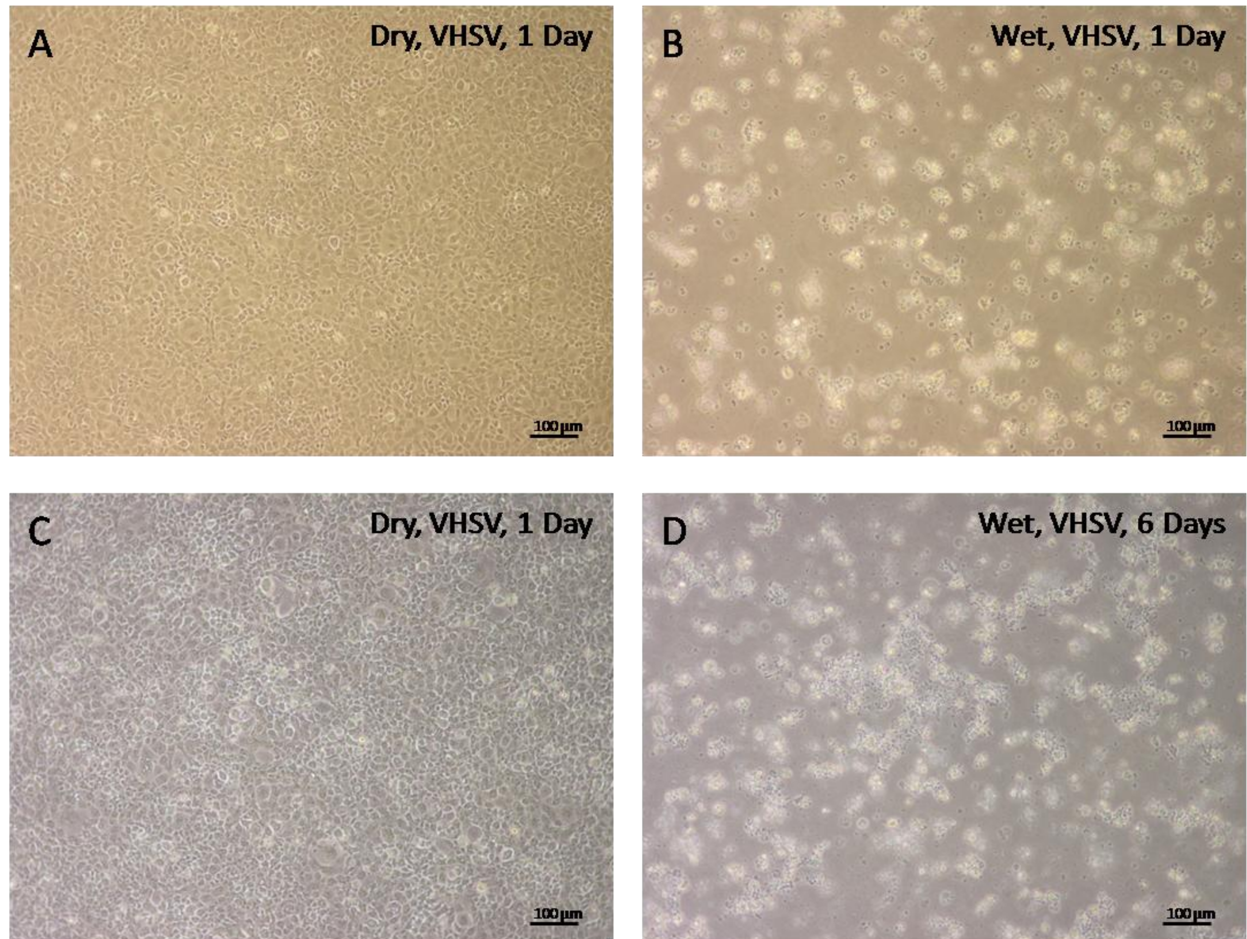

Figure 2- 3. The capacity of fishing hooks to transfer infectious VHSV.

Danielson (A and B) and Mustad (C and D) brand fishing hooks were inoculated with 400 µl of stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) in 10 ml of 2% FBS/L15 for 24 hours at 14°C. All hooks were then rinsed with D-PBS. One set from each brand (A and C) was left to dry at room temperature for 10 day, while 10 ml of fresh 2% FBS/L15 was added to the other set in each brand (B and D) which were left at room temperature 1 day (B) and 6 days (D). After each incubation time, a hook from each of the dry set (A and C) and the wet set (B and D) were separately added to a dish containing a monolayer of EPC cells. All cultures were then incubated at 14°C for 7 to 10 days prior to being photographed at 100 X with a camera mounted on an inverted phase-contrast microscope. Hooks that were left to dry for even only 1 day (A and C) did not retain any infectious VHSV as the EPC monolayers that were inoculated by those hooks remained intact. Danielson hook incubated wet for one day (B) and Mustad hook incubated wet for 6 days (D) retained infections VHSV that completely destroyed the EPC monolayers. EPC monolayers inoculated with control hooks remained intact (data not shown). The scale bar shows 100 µm.

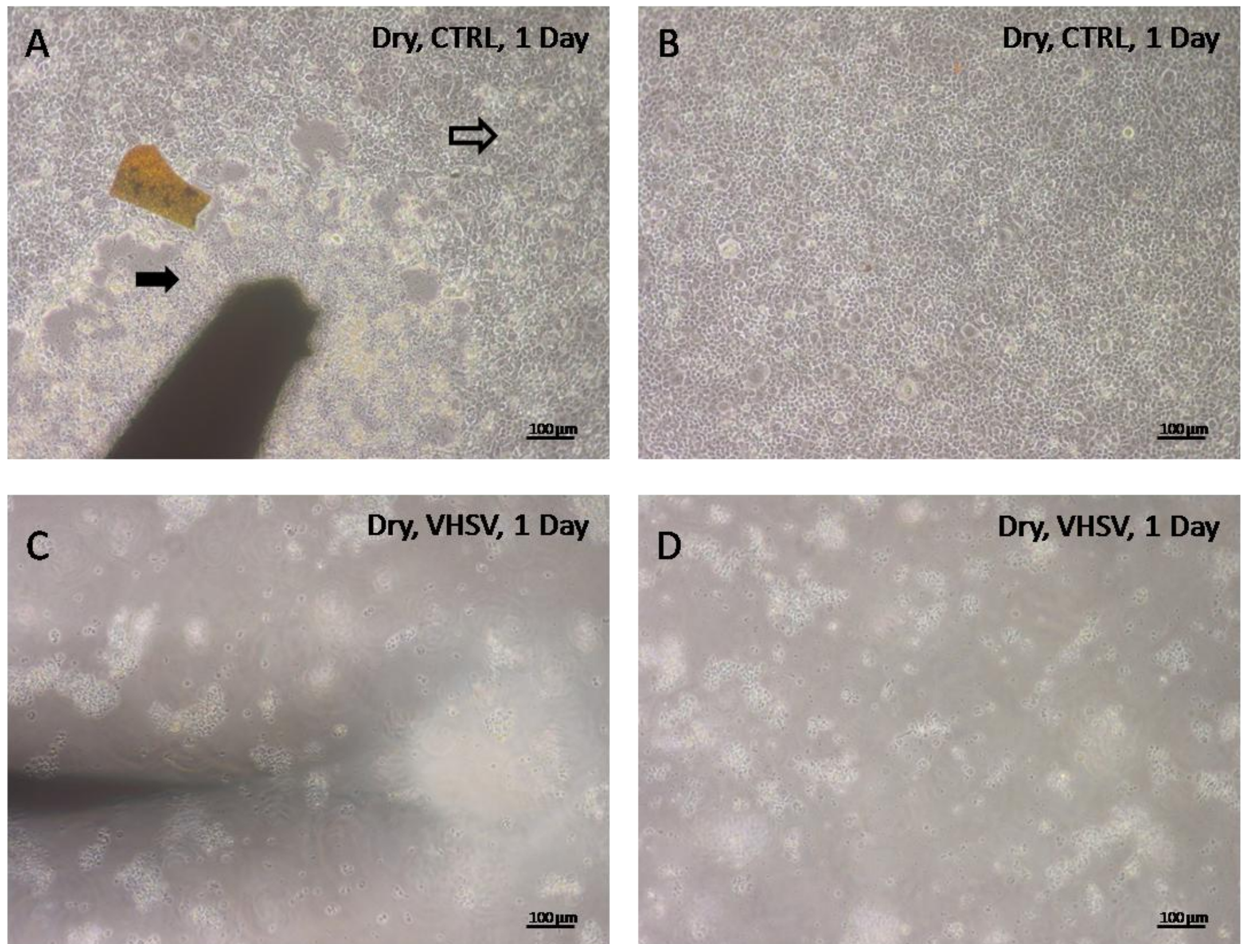

Figure 2- 4. The appearance of rust from hooks on reporter cell monolayer.

Mustad brand (A and C) fishing hooks were inoculated with 400 µl of 2% FBS/L15 with no VHSV and (B and C) were inoculated with 400 µl of stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) in 10 ml of 2% FBS/L15 for 24 hours at 14°C. All hooks were then rinsed with D-PBS and left to dry at room temperature for 1 day. After 1 day, all hooks were separately added to a dish containing a monolayer of EPC cells. All cultures were then incubated at 14°C for 7 to 10 days prior to being photographed at 100 X with a camera mounted on an inverted phase-contrast microscope. In A, the control hooks that had no virus produced rust that killed nearby EPC cells in a concentration dependent manner (solid arrow). Cells that were far from the hook did not die (hollow arrow). B shows a different, completely intact, area of the same monolayer as in A. In C, the EPC monolayer showed complete destruction that is not in a concentration dependent manner. D shows complete destruction of the same monolayer as in C but much farther away from the hook. Dark area in the images shows the tip of the hooks. The scale bar shows 100 µm.

3.3.3 Fishing lines

Two different types of fishing lines were exposed to VHSV and then dried at room temperature for up to 10 days. The Northern Sport Braid line was consistently able to transfer infectious VHSV to reporter cell monolayer when left to dry for one day (Figure 2-5, Panel A). The Spiderwire Mono fishing line was also able to transfer infectious VHSV when left to dry for one day (Panel C) but not consistently (Table 4). Neither fishing line was able to transfer infectious VHSV to reporter culture when left to dry for 6 days. When the fishing lines were incubated in wet condition at room temperature after VHSV exposure, both lines were able to transfer infectious VHSV to reporter cell monolayer even by the 10th day of incubation (Panels B and D).

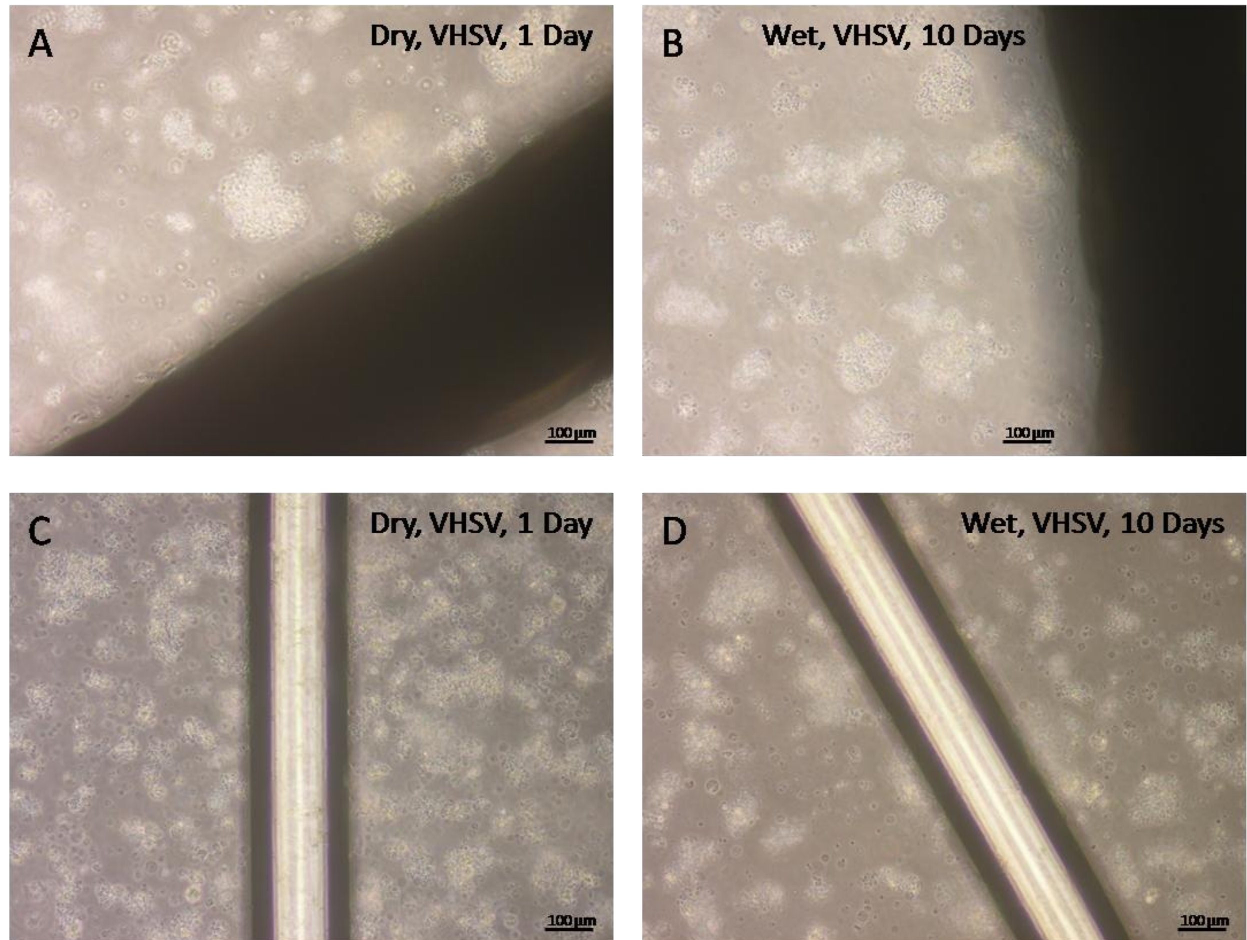

Figure 2- 5. The capacity of fishing lines to transfer infectious VHSV.

Northern Braid (A and B) and Spiderwire (C and D) brand fishing lines were inoculated with 400 µl of stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) in 10 ml of 2% FBS/L15 for 24 hours at 14°C. All fishing lines were then rinsed with D-PBS. One set from each brand (A and C) was left to dry at room temperature for 1 day, while 10 ml of fresh 2% FBS/L15 was added to the other set in each brand (B and D) which was left at room temperature for 10 days. After each incubation time, a line from each of the dry sets (A and C) and the wet sets (B and D) were separately added to dishes containing a monolayer of EPC cells. All cultures were then incubated at 14°C for 7 to 10 days prior to being photographed at 100 X with a camera mounted on an inverted phase-contrast microscope. Fishing lines that were left to dry for 1 day (A and C) and those that were incubated wet for 10 days (B and D) carried infectious VHSV leading to the destruction of the EPC monolayers that those lines were inoculated on. EPC monolayers inoculated with control fishing lines remained intact (data not shown). The Northern Braid fishing lines can be seen as dark area in A and B, and the Spiderwire lines can be seen as the clear line across the images in C and D. The scale bar shows 100 µm.

3.3.4 Commercial soft drink can and water bottle pieces

A mini Sprite soft drink can and a Nestle PureLife water bottle was cut into pieces about 1 cm^2 in size and exposed to VHSV. After being rinsed with D-PBS, the pieces were either left to dry at room temperature or incubated in L15 with 2% FBS for up to 10 days. When left to dry, pop can pieces were able to transfer infectious VHSV after one day (Figure 2-6, Panel A) but lost the capacity by the 6th day (Table 4). Water bottle pieces were able to transfer infectious VHSV after one and six days (Panel C), however; the result of the 6th day is inconsistent (Table 4). When incubated in wet condition, both the pop can and water bottle pieces were able to transfer VHSV even by the 10th day of incubation (Panels B and D).

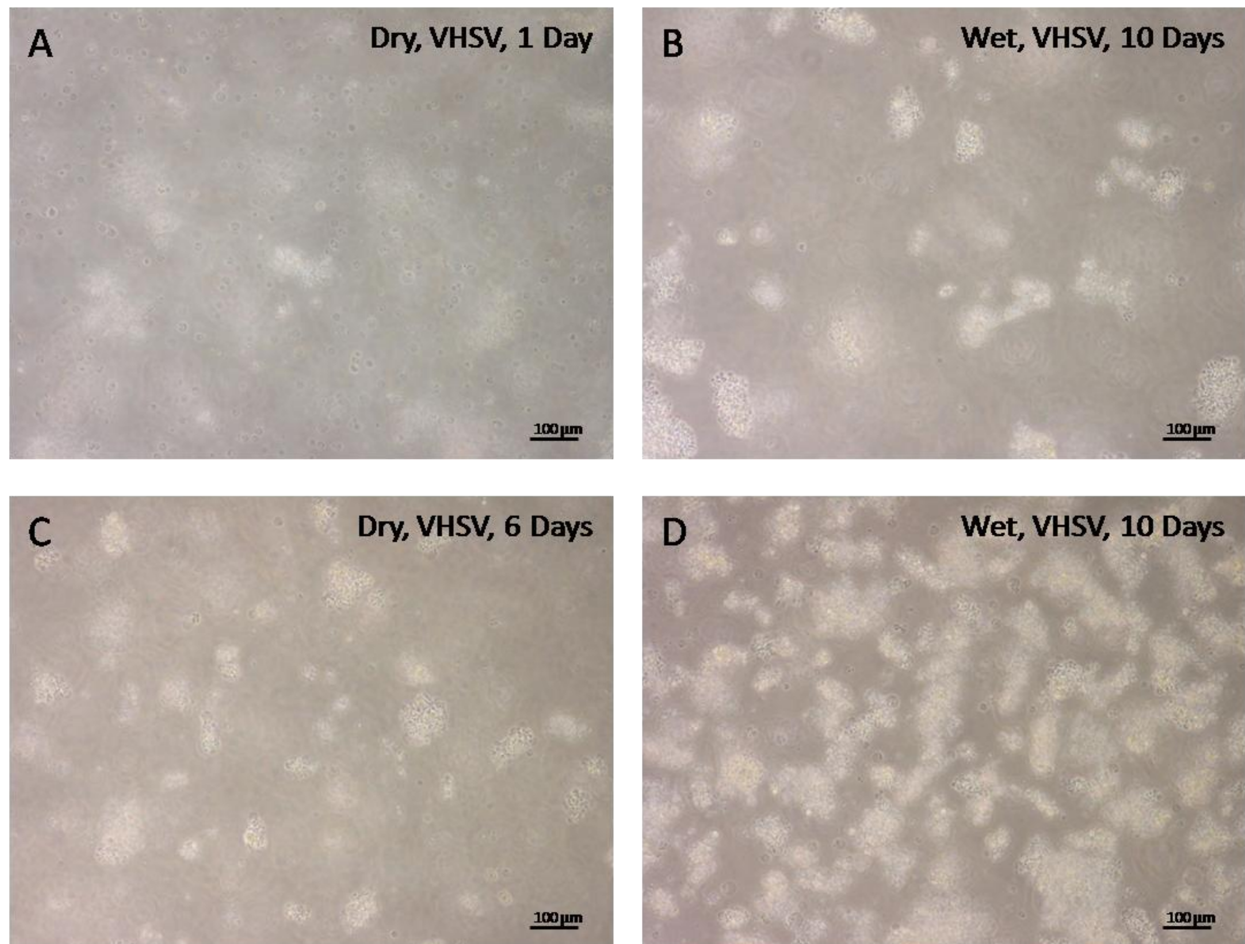

Figure 2- 6. The capacity of commercial soft drink (pop) can water bottle to transfer infectious VHSV.

Pop can pieces (A and B) and water bottle pieces (C and D) were inoculated with 400 µl of stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) in 10 ml of 2% FBS/L15 for 24 hours at 14°C. All pieces were then rinsed with D-PBS. One set of the pop can pieces (A) and water bottle pieces (C) were left to dry at room temperature for 1 day (A) and 6 days (C). 10 ml of fresh 2% FBS/L15 was added to the other set of pop can (B) and water bottle (D) pieces which was left at room temperature for 10 days. After each incubation time, a piece from each of the dry sets (A and C) and the wet sets (B and D) were separately added to dishes containing a monolayer of EPC cells. All cultures were then incubated at 14°C for 7 to 10 days prior to being photographed at 100 X with a camera mounted on an inverted phase-contrast microscope. In A and C, EPC monolayers were destroyed by infectious VHSV from pop can pieces left to dry for 1 day (A) and water bottle pieces left to dry for 6 days (C). In B and D, EPC monolayers were destroyed by infectious VHSV from pop can pieces (B) and water bottle pieces (D) that were incubated wet in 2% FBS/L15 for 10 days. EPC monolayers inoculated with control pop can or water bottle pieces remained intact (data not shown). The scale bar shows 100 µm.

3.4 Examining VHSV attachment and survival on various objects using the surface-to-cell transfer (SCT) method

Inasmuch as VHSV survived on inanimate objects for only a day as detected by either ET or OAT, another approach was sought to determine whether the virus could survive on dry surfaces for longer periods and has been named surface-to-cell transfer (SCT) of virus. The idea of SCT is that cells can attach, form a monolayer, and become infected on surfaces on which viruses have been dried. In order for SCT to be monitored, the surface should support the attachment and spreading of the reporter cells, be transparent, and be on an object with dimensions that allow it to be viewed with an inverted phase contrast microscope so that the development of CPE can be seen. SCT was briefly shown in “Dishes” column of Table 3 in the ET section. This demonstrates that EPC cells can become infected upon attaching and spreading on plastic surfaces on which VHSV has been dried. Surface-bound virus can be envisioned as either directly binding cell receptors to initiate an infection and/or to be eluted first into the cell culture medium from which the virus binds cell receptors to cause an infection. Therefore the principle of SCT of virus has been demonstrated.

As a test of whether SCT was more sensitive than ET and OAT, dishes and 6-well plate cell culture inserts were prepared with surface-bound VHSV and allowed to dry at room temperature for up to 15 days for the dishes and 6 days for the cell culture inserts. CPE developed in plastic dishes that had been dried for up to 15 days and in glass dishes and 6-wells plate inserts that had been dried for up to 6 days (Figure 2-7). With the ET approach, very small amount of infectious VHSV was eluted after 6 days of drying and with the OAT approach, the survival of VHSV could be demonstrated for only 1 day of drying on both surfaces. Therefore, SCT is superior for detecting VHSV on dry surfaces.

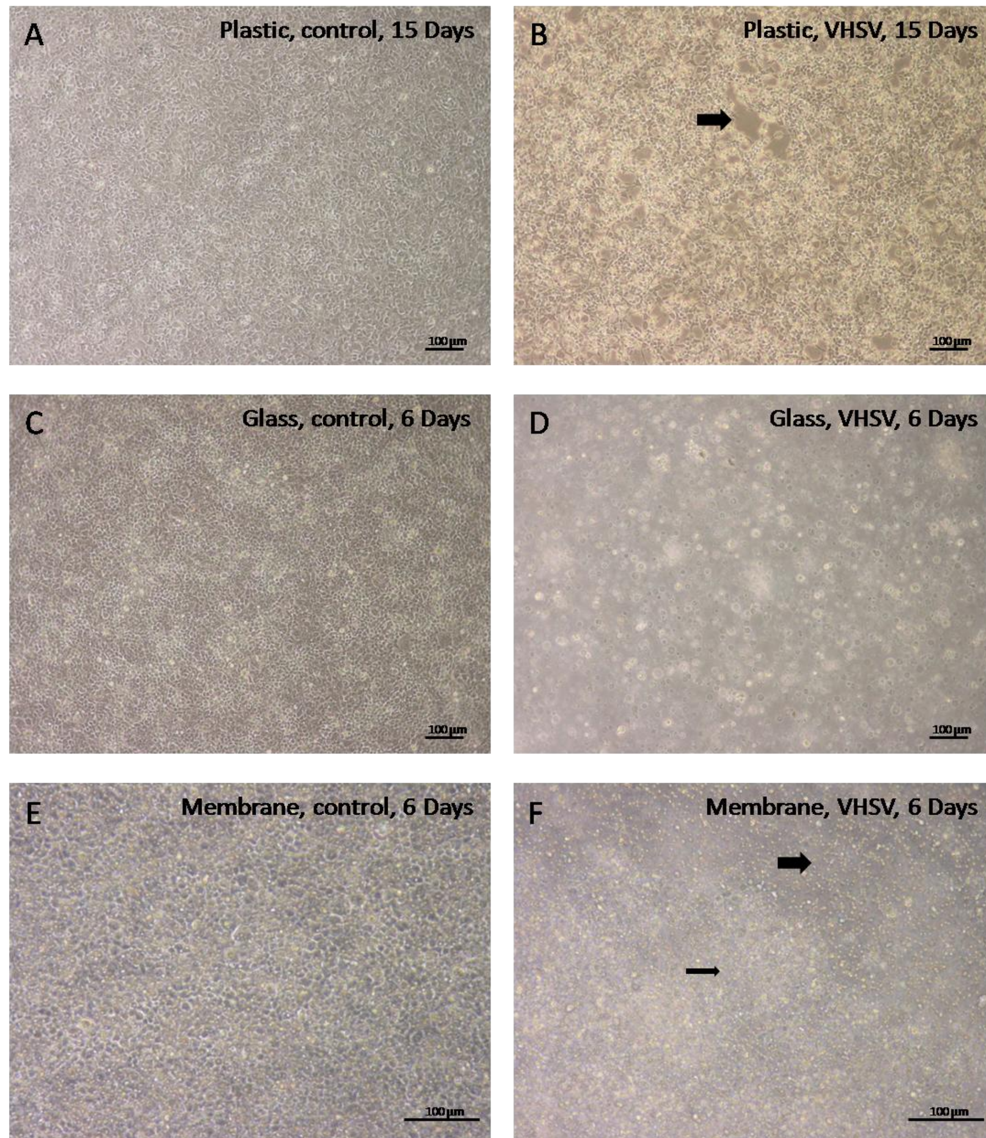

Figure 2- 7. Survival of VHSV on plastic and glass dishes and 6-well plate inserts at room temperature dried beyond 1 day.

6 ml of 2 % FBS/L-15 without (A, C and E) or with 400 µl of VHSV at $10^{8.8}$ $TCID_{50/200\ ul}$ (B, D and F) were added to each of two 60 mm plastic tissue culture dishes (A and B), two 60 mm soda lime glass dishes (C and D) and two 6-wells plate inserts with porous membrane as the base (E and F). The plastic dishes (A and B) were allowed to dry for 15 days at room temperature and the glass dishes (C and D) and inserts (E and F) were allowed to dry for 6 days at room temperature. After the dishes and inserts had been incubated for these times, approximately 2.6 x 10^5 EPC cells in 2 % FBS/L-15 were added per dish and insert. All cultures were then incubated at 14°C for 7 to 10 days prior to being photographed at 100 X for dishes and 200 X for inserts with a camera mounted on an inverted phase-contrast microscope. In A, C and E, the control dishes and inserts, a monolayer of cells covers the surface. In B, the monolayer shows a small degree of destruction resulting in cell-free regions of plastic surface in the monolayer (arrow), which is the CPE. In D and F, the monolayer was completely destroyed leaving floating cellular debris and rounded cells to represent CPE. The thin arrow in F illustrates floating debris and the thick arrow shows cell-free regions of insert membrane surface. The pores are visible as small dots in F, whereas in E they are covered by cell monolayer. The scale bar shows 100 µm.

3.4.1 The Survival of VHSV left to dry on 60 mm plastic dishes at various temperatures

When VHSV was left to dry on plastic dishes at 4°C, 14°C and room temperature, the virus was able to survive for up to 15 days. Panels B, C and D in Figure 2-8 shows the presence of CPE in the reporter cells monolayer that was plated on polystyrene plastic dishes containing VHSV that was left to dry for 15 days at 4°C, 14°C and room temperature respectively. When the drying temperature was increased to 26°C, VHSV did not survive to the 6th day time point and beyond. The reporter cells that were plated on dishes containing VHSV dried for 6 or more days formed a monolayer that did not show any CPE (Table 5). However, one day of drying at 26°C was not enough to inactivate all VHSV on the plastic dish (Panel E). VHSV also managed to survive being dried on a plastic dish at 37°C for 1 day and caused CPE in reporter cells that were subsequently added to the dish (Panel F). The summary of all results for VHSV survival on polystyrene plastic at all five incubation temperatures and all time points is listed in Table 5. Figure 2-8 only show selected results from Table 5.

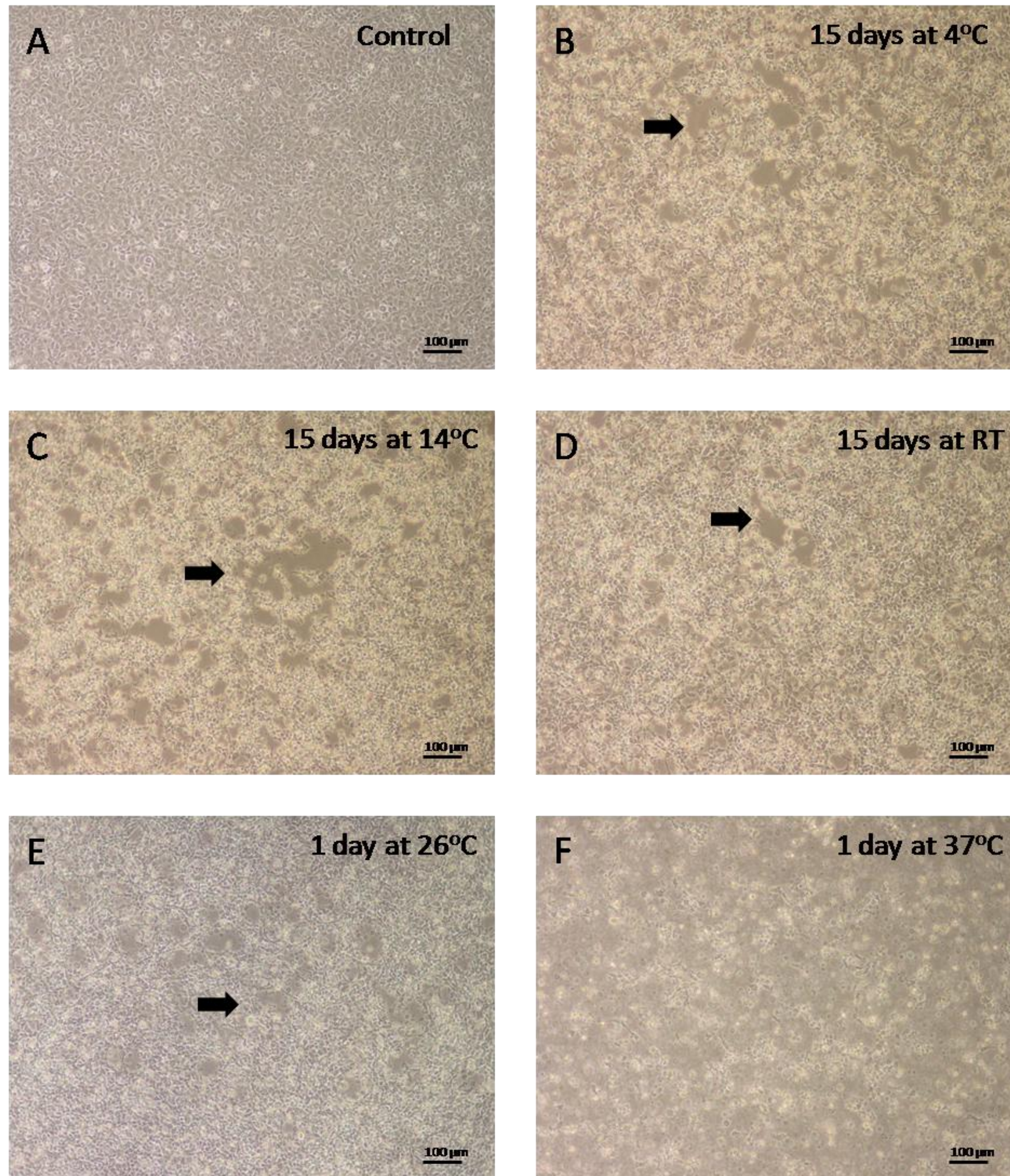

Figure 2- 8. Survival of VHSV dry on plastic dishes at different temperatures.

6 ml of 2 % FBS/L-15 without (A) or with 400 µl of VHSV at $10^{8.8}$ $TCID_{50/200\ ul}$ (B to F) were added to each of six 60 mm plastic tissue culture dishes which were then allowed to dry for 15 days at 4 °C (B), 14 °C (C) and room temperature (RT) (D) or for 1 day at 26 °C (E) and 37 °C (F). The image in panel D was previously shown in Figure 2-7 above but is repeated here for comparison purposes with other incubation temperature. The representative control (A) was allowed to dry at 4°C for 15 days. Controls at other temperatures are not shown. After the dishes had been incubated for these times at these temperatures, approximately 2.6 million EPC cells in 2 % FBS/L-15 were added per dish. All cultures were then incubated at 14°C for 7 to 10 days prior to being photographed at 100 X with a camera mounted on an inverted phase-contrast microscope. In A, a monolayer of cells covers the surface, whereas in B to F, the monolayer shows various degrees of destruction, which is the CPE. At this level of magnification, cultures with CPE have floating cellular debris, round cells, and cell-free regions of plastic surface (arrows). The scale bar shows 100 µm. This figure shows images of select results from Table 5.

Table 5. Survival of VHSV dry on 60 mm Petri dish surfaces at different temperatures and times.

Type of Petri dish	Occurrence of CPE* 10 days after adding EPC cells** to Petri dishes				
	Dish incubation conditions after adding VHSV*** and prior to adding EPC				
	Temperature	Time in days			
		1	6	10	15
Plastic					
	4°C	+	+	+	+
	14°C	+	+	+	+
	RT****	+	+	+	+
	26°C	+	-	-	-
	37°C	+	-	-	-
Glass					
	4°C	+	+	+	+
	14°C	+	+	-	-
	RT****	+	+	-	-
	26°C	+	-	-	-
	37°C	-	-	-	-

*CPE stands for cytopathic effect and is indicated as either occurring (+) or not (-).
** EPC stands for a carp cell line
*** 6 ml of 2 % FBS/L-15 without (A) or with 400 µl of VHSV at $10^{8.8}$ $TCID_{50/200\ ul}$ were added per Petri dish (60 mm) and incubated as described.
****RT stands for room temperature.

3.4.2 The survival of VHSV left to dry on 60 mm glass dishes at various temperatures

VHSV was able to survive being dried on glass for up to 15 days at 4°C (Figure 2-9, Panel B). When dried on glass at 14°C and room temperature, VHSV was only able to survive for up to 6 days (Panels C and D, respectively). The reporter cells showed no CPE when plated into dishes that was dried with VHSV for 10 or more days at 14°C and room temperature (Table 5). At 26°C, VHSV survived drying for only 1 day (Panel E). By the 6th day and beyond, all VHSV in the 26°C dried dishes were inactivated resulting in no CPE present in reporter cells (Table 5). When the drying temperature was 37°C, VHSV was completely inactivated on glass dishes after only 1 day. Reporter cells plated into a dish containing VHSV that was dry for 1 day at 37°C showed no CPE (Panel F). The summary of all results for VHSV survival on glass at all five incubation temperatures and all time points is listed in Table 5. Figure 2-9 only show selected results from Table 5.

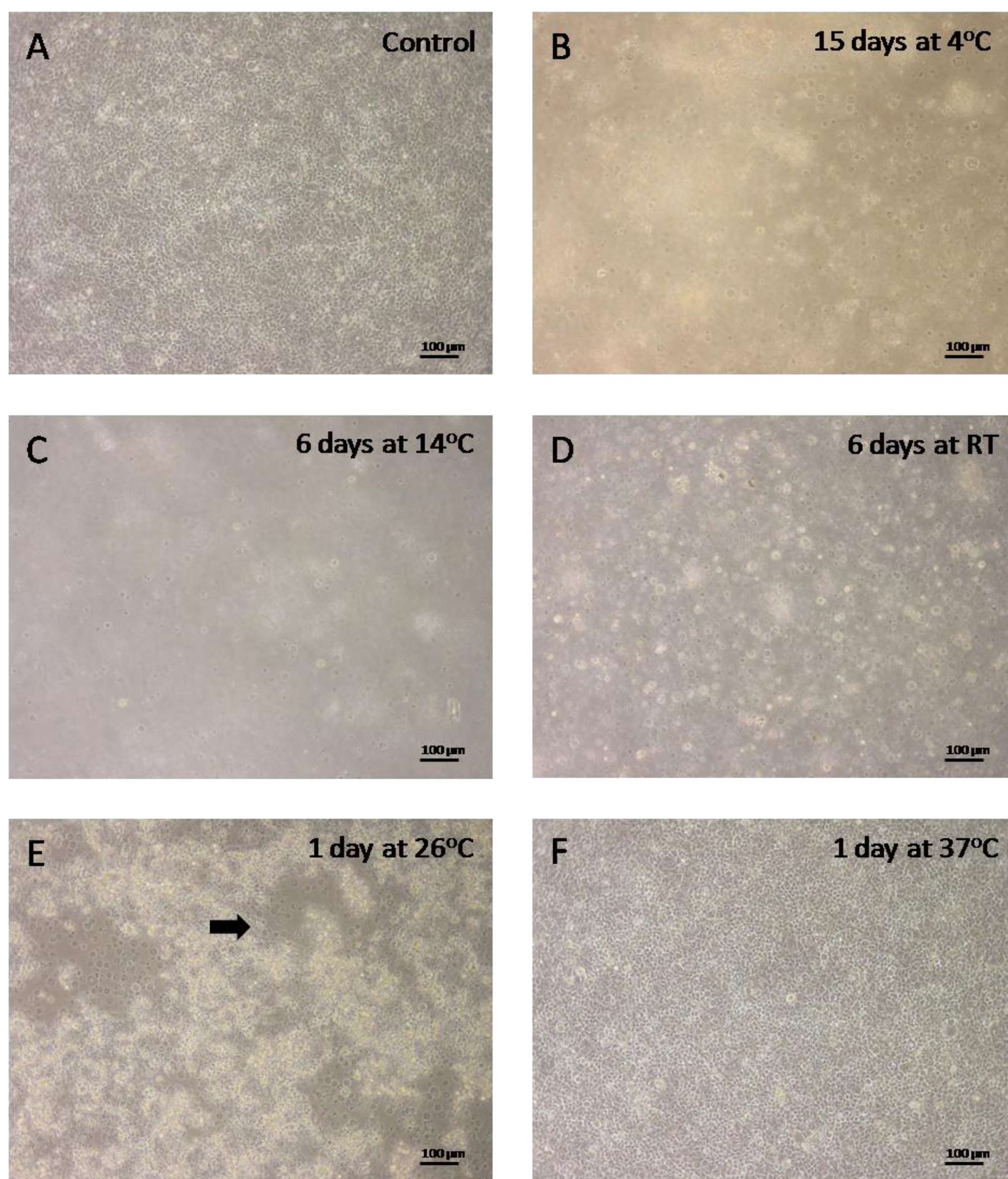

Figure 2- 9. Survival of VHSV dry on glass dishes at different temperature.

6 ml of 2 % FBS/L-15 without (A) or with 400 µl of VHSV at $10^{8.8}$ $TCID_{50/200\ ul}$ (B to F) were added to each of six 60 mm glass culture dishes which were then allowed to dry for 15 days at 4 °C (B), 14 °C (C) and room temperature (D) or for 1 day at 26 °C (E) and 37 °C (F). The image in panel D was previously shown in Figure 2-7 above but is repeated here for comparison purposes with other incubation temperature. The representative control (A) was allowed to dry at 4°C for 6 days. Controls at other temperatures are not shown. After the dishes had been incubated for these times at these temperatures, approximately 2.6 million EPC cells in 2 % FBS/L-15 were added per dish. All cultures were then incubated at 14°C for 7 to 10 days prior to being photographed at 100 X with a camera mounted on an inverted phase-contrast microscope. In A (the control) and F (1 day at 37 °C), a monolayer of cells covers the surface, whereas in B to F, the monolayer shows various degrees of destruction, which is the CPE. At this level of magnification, cultures with CPE have floating cellular debris, round cells, and small regions free of cells (arrow in E) or the glass surface is completely free of cells (C). The scale bar shows 100 µm. This figure shows images of select results from Table 5.

3.5 Relative quantitative comparison of VHSV inactivation using 96-well microtiter plate SCT method

An attempt to quantify SCT of virus was made through the use of 96-well tissue culture plates, which is referred to as the 96-well method and used to study the effect of drying time and temperature on the survival of VHSV. A way of quantifying SCT of virus was sought because the procedure with Petri dishes records only whether all the virus was inactivated (-) or whether some unknown fraction was still active (+) and fails to indicate whether a proportion of the virus is inactivated. One approach would be to take samples from different sites of the same Petri dish surface and to evaluate each sample as a positive/negative and to express the proportion of positive samples. However, sampling different sites of identical areas on a Petri dish is difficult so instead a 96-well plastic tissue culture plates was coated with VHSV and considered a single surface. Each well of a 96-well plate is isolated from each other so virus that remains active in one well will not infect adjacent wells. Therefore, by counting the number of wells per plate with active VHSV, the relative amount of VHSV survival can be compared between different experimental conditions when using 96-well plates. For each plate, VHSV was exposed to only 80 wells (column 3 to 12 on the plate). Column 1 and 2 of each plate, 16 wells in total, were not exposed to VHSV and served as control wells. For each drying condition (temperature and time), two 96-well plates were used. Each plate was exposed to the virus under identical conditions and rinsed. To one plate (wet), medium was added back to each well. To the second plate (dry), nothing was added. Both were incubated for the same length of time and temperature before EPC cells were added to all wells per plate to indicate ten days later the proportion positive/negative of VHSV exposed wells with CPE. Wells with CPE were identified by their diminished capacity to reduce Alamar Blue. Wells positive for CPE indicate the occurrence of SCT. The two types of plates provide a relative quantitative measure of the ability of VHSV to survive on a plastic surface in either the dry or wet state.

As a test of the sensitivity of the 96-well plate method to report the dry survival of VHSV on tissue culture plastic, different dilutions of virus were applied to 96-well plates and dried at room temperature for up to 15 days. For each dilution of virus from 10^{-1} to 10^{-5} of stock ($10^{8.8}$ $TCID_{50/200\ ul}$) and for each drying period (1, 6, 10 and 15 days), a single 96-well plate was used. Afterwards, EPC cells were added to 80 wells for each plate and these were scored for the presence or absence of CPE 10 days later. The 96-well SCT method was not able to detect differences in VHSV inactivation when the initial seeded virus was in the dilution range from 10^{-1}

to 10^{-4}. There was maximum VHSV survival (80 wells with active VHSV) as defined by the method within that dilution range by the 10th day of being dried (Figure 2-10). When VHSV was further diluted to 10^{-5}, the 96-well plate method was able to detect differences in VHSV inactivation relative to the other dilutions but only at the 10th day. By the 10th day of drying, less than an average of 60 wells retained active VHSV (Figure 2-10). At the 15th day of drying, there was complete inactivation of VHSV at all of the initial seeded viral dilutions. Thus, SCT of virus can be evaluated in 96-well plastic plates as the proportion of positive wells.

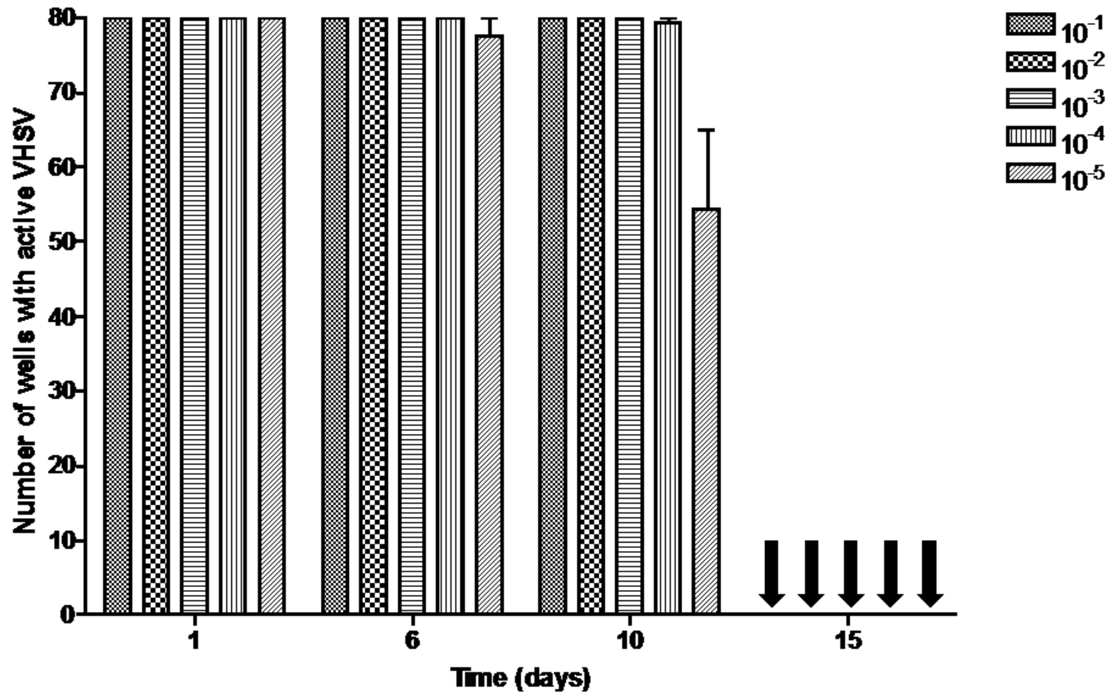

Figure 2- 10. The survival of various dilutions of VHSV dried at room temperature on 96-well plate.

Stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) was serially diluted in L15 with 2% FBS to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions. Each dilution of VHSV was seeded into 80 wells of four 96-wells plate at a total volume of 100 µl per well. L15 with 2% FBS was added to 16 control wells of each plate. All plates were rinsed with D-PBS and then dried at room temperature for up to 15 days. At day 1, 6, 10 and 15 time points, 50 000 EPC cells were added to each well of a plate from each dilution. After addition of reporter cells, the plates were incubated at 14°C for 7 to 10 days and then scored with Alamar Blue for reporter cell viability. Death of cells in VHSV exposed wells but not in control wells indicate the presence of active VHSV. All 80 exposed well were scored for presences of active VHSV and the total number of wells with active VHSV per plate was tallied. For all dilutions except (10^{-5}), there was maximum number of wells with active VHSV by the 10th day of drying. For the 10^{-5} dilution, about one third of the wells were not infective. By the 15th day of drying, there was no active VHSV reported in any of the wells at all dilutions (arrows).

3.5.1 Relative quantitative comparison of VHSV survival at 4°C in dry and wet environments

Over the 15 days drying period at 4°C, very little VHSV inactivation occurred. VHSV left to dry for one day at 4°C resulted in no inactivation as detectable by the 96-well plate method. All 80 VHSV exposed wells consistently showed total reporter cell death as measured by Alamar Blue (Figure 2-11) therefore giving a maximum of 80 wells with active VHSV. The average amount of VHSV survival decreased as the number of days left to dry increased. By the 15th day of drying, the average number of wells showing active VHSV was 57 out of the 80 initially exposed well. However, this decrease in active VHSV is not always consistent at 4°C as some experimental repeat showed all 80 wells with active virus, leading to a high standard deviation (Figure 2-11). When VHSV was incubated in L15 with 2% FBS, the wet condition, at 4°C there was no detectable decrease in active virus over the 15 days incubation period using the 96-well plate method (Figure 2-11).

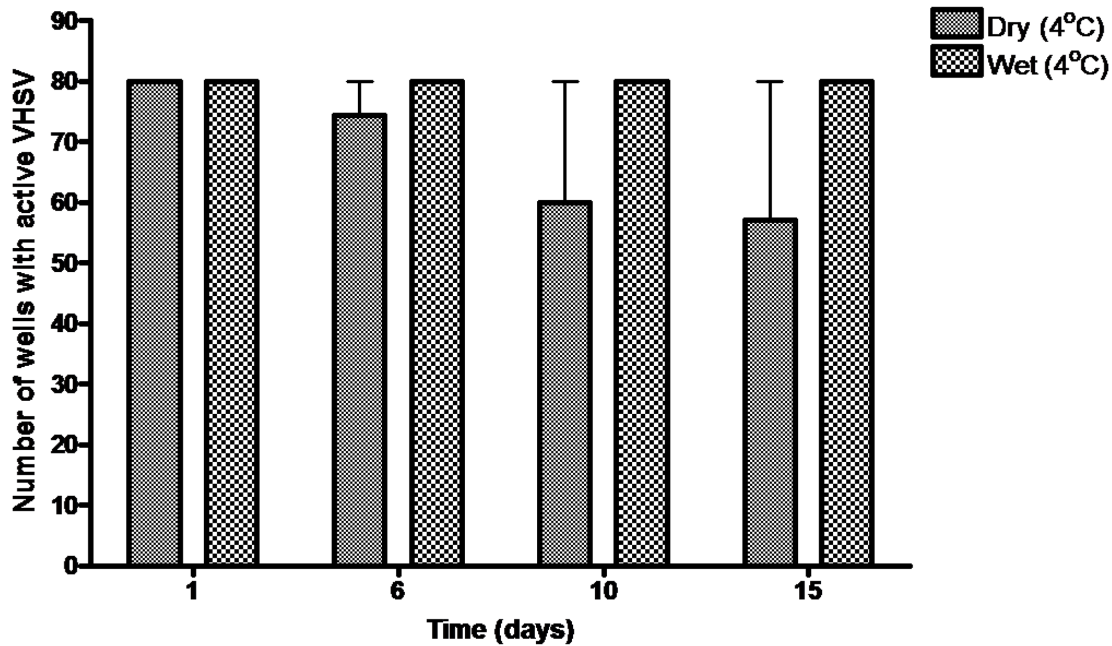

Figure 2- 11. Survival of VHSV on 96-well plates in dry and wet condition at 4°C.

When dried at 4°C, the average number of wells with active VHSV decreased as the incubation time increased. However, there was no decrease in the average number of wells with active VHSV when incubated in wet condition. Stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) was diluted in L15 with 2% FBS to 10^{-2} and was seeded into 80 wells of four 96-wells plate at a total volume of 100 µl per well for each of the dry and wet condition. L15 with 2% FBS was added to 16 control wells of each plate at a total volume of 100 µl per well. All plates were rinsed with D-PBS. After rinsing, nothing was further added to the plates in the dry condition but 100 µl of L15 with 2% FBS was added to the plates in the wet condition. All plates were incubated at 4°C for up to 15 days. At day 1, 6, 10 and 15 time points, 50 000 EPC cells were added to each well of a plate from both conditions. After addition of reporter cells, the plates were incubated at 14°C for 7 to 10 days and then scored with alamar Blue for reporter cell viability. Death of cells in VHSV exposed wells but not in control wells indicate the presence of active VHSV.

3.5.2 Relative quantitative comparison of VHSV inactivation at 14°C in dry and wet environments

There is a steady decrease in the amount of active VHSV left to dry at 14°C over 15 days. VHSV left to dry for one day at 14°C resulted in no inactivation detectable by 96-well plate method. All 80 VSHV exposed wells consistently showed complete reporter cell death as measured by Alamar Blue (Figure 2-12) therefore giving a maximum of 80 wells with active VHSV. The average amount of VHSV survival decreased as the number of days left to dry increased. Even taking into account the high standard deviation between replicates, there is still a definite decrease in the amount of active VHSV by the 10th day of drying and beyond (Figure 2-12). By the 15th day of drying, only about a quarter of the 80 initially exposed wells showed active VHSV. When VHSV was incubated in L15 with 2% FBS, the wet condition, at 14°C there was no detectable decrease in active virus over the 15 days incubation period (Figure 2-12).

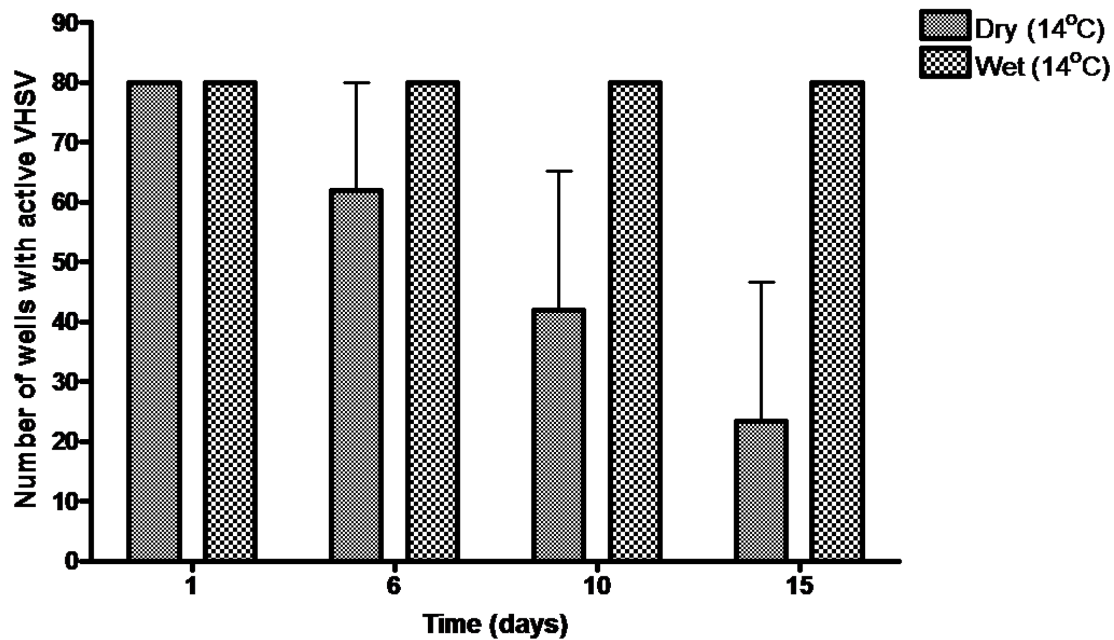

Figure 2- 12. Survival of VHSV on 96-well plates in dry and wet state at 14°C.

When dried at 14°C, the average number of wells with active VHSV decreased as the incubation time increased. However, there was no decrease in the average number of wells with active VHSV when incubated in wet condition. Stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) was diluted in L15 with 2% FBS to 10^{-2} and was seeded into 80 wells of four 96-wells plate at a total volume of 100 µl per well for each of the dry and wet condition. L15 with 2% FBS was added to 16 control wells of each plate at a total volume of 100 µl per well. All plates were rinsed with D-PBS. After rinsing, nothing was further added to the plates in the dry condition but 100 µl of L15 with 2% FBS was added to the plates in the wet condition. All plates were incubated at 14°C for up to 15 days. At day 1, 6, 10 and 15 time points, 50 000 EPC cells were added to each well of a plate from both conditions. After addition of reporter cells, the plates were incubated at 14°C for 7 to 10 days and then scored with Alamar Blue for reporter cell viability. Death of cells in VHSV exposed wells but not in control wells indicate the presence of active VHSV.

3.5.3 Relative quantitative comparison of VHSV inactivation at room temperature in dry and wet environments

When VHSV was left to dry in a 96-well plate at room temperature over 15 days, there is about a one third decrease in active VHSV at each of the sampled time point (Figure 2-13). VHSV left to dry for only one day at room temperature resulted in no inactivation detectable by 96-well plate method. All 80 VSHV exposed wells consistently showed complete reporter cell death as measured by Alamar Blue (Figure 2-13) therefore giving a maximum of 80 wells with active VHSV. By the 10th day of drying, only about a little more than a quarter of the 80 initially exposed wells showed active VHSV. By the 15th day, there was no active VHSV left in any wells of the 96-well plate. When VHSV was incubated in L15 with 2% FBS, the wet condition, at room temperature there was no detectable decrease in active virus over the 15 days incubation period using the 96-wells plate method (Figure 2-13).

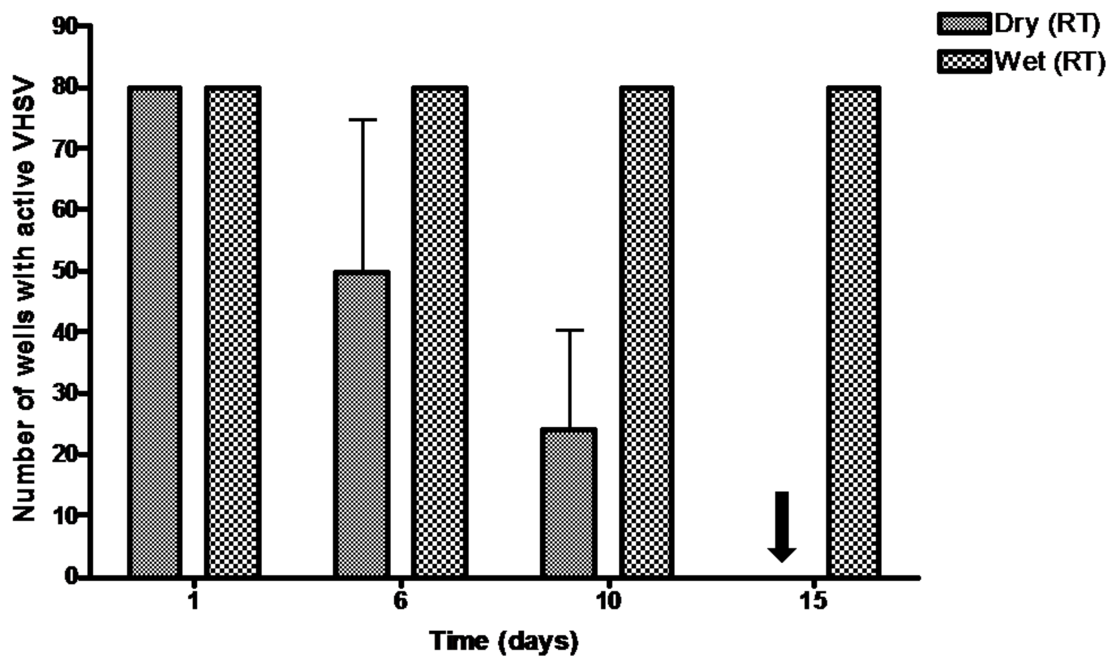

Figure 2- 13. Survival of VHSV on 96-well plates in dry and wet state at room temperature.

When dried at room temperature (RT), the average number of wells with active VHSV decreased as the incubation time increased. The arrows indicate no VHSV survival, no wells with active VHSV, at the 15th day of drying. However, there was no decrease in the average number of wells with active VHSV when incubated in wet condition. The arrow indicates no VHSV survival, no wells with active VHSV, at the 15th day. Stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) was diluted in L15 with 2% FBS to 10^{-2} and was seeded into 80 wells of four 96-wells plate at a total volume of 100 µl per well for each of the dry and wet condition. L15 with 2% FBS was added to 16 control wells of each plate at a total volume of 100 µl per well. All plates were rinsed with D-PBS. After rinsing, nothing was further added to the plates in the dry condition but 100 µl of L15 with 2% FBS was added to the plates in the wet condition. All plates were incubated at room temperature for up to 15 days. At day 1, 6, 10 and 15 time points, 50 000 EPC cells were added to each well of a plate from both conditions. After addition of reporter cells, the plates were incubated at 14°C for 7 to 10 days and then scored with Alamar Blue for reporter cell viability. Death of cells in VHSV exposed wells but not in control wells indicate the presence of active VHSV.

3.5.4 Relative quantitative comparison of VHSV inactivation at 26°C in dry and wet environments

VHSV left to dry for only one day at 26°C resulted in a slight inactivation of VHSV as detectable by 96-well plate method. However, this inactivation is not always consistent between replicates. By the 6th day of drying, only about a quarter of the 80 initially exposed wells showed active VHSV. By the 10th day and beyond, there was no active VHSV left in any wells of the 96-well plate. When VHSV was incubated in L15 with 2% FBS, the wet condition, at 26°C was no detectable decrease in active virus over the 15 days incubation period using the 96-well plate method (Figure 2-14).

3.5.5 Relative quantitative comparison of VHSV inactivation at 37°C in dry and wet environments

VHSV left to dry for only one day at 37°C resulted in near complete inactivation as detectable by 96-well plate method. There remains near a quarter of infectious VHSV when incubated at 37°C in L15 with 2% FBS for one day, about twice the amount that survived when compared to the dry condition. When VHSV was incubated at 37°C for 6 days and beyond, in both dry and wet conditions, all virus was inactivated (Figure 2-15).

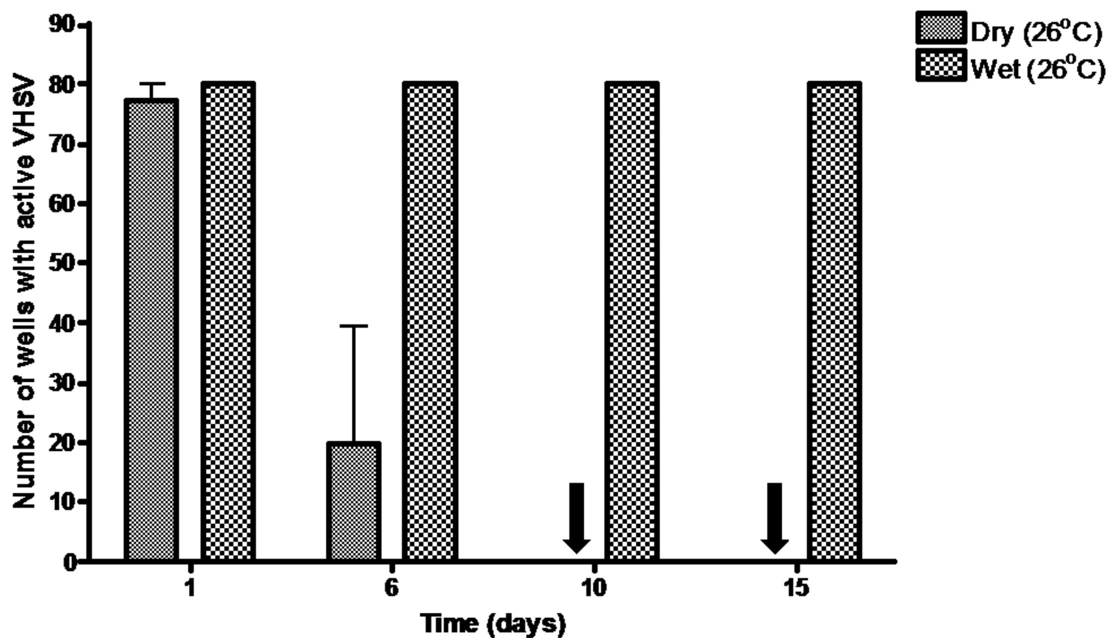

Figure 2- 14. Survival of VHSV on 96-well plates in dry and wet state at 26°C.

When dried at 26°C, the average number of wells with active VHSV decreased as the incubation time increased. The arrows indicate no VHSV survival, no wells with active VHSV, at the 10th and 15th day of drying. However, there was no decrease in the average number of wells with active VHSV when incubated in wet condition. Stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) was diluted in L15 with 2% FBS to 10^{-2} and was seeded into 80 wells of four 96-wells plate at a total volume of 100 µl per well for each of the dry and wet condition. L15 with 2% FBS was added to 16 control wells of each plate at a total volume of 100 µl per well. All plates were rinsed with D-PBS. After rinsing, nothing was further added to the plates in the dry condition but 100 µl of L15 with 2% FBS was added to the plates in the wet condition. All plates were incubated at 26°C for up to 15 days. At day 1, 6, 10 and 15 time points, 50 000 EPC cells were added to each well of a plate from both conditions. After addition of reporter cells, the plates were incubated at 14°C for 7 to 10 days and then scored with Alamar Blue for reporter cell viability. Death of cells in VHSV exposed wells but not in control wells indicate the presence of active VHSV.

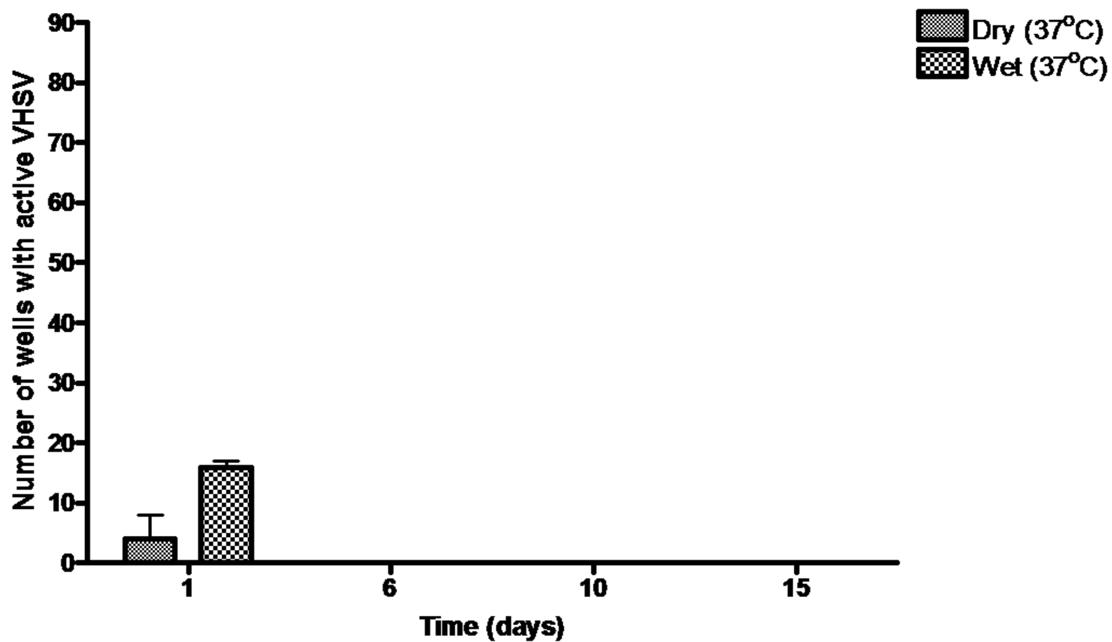

Figure 2- 15. Survival of VHSV on 96-well plates in dry and wet state at 37°C.

The absence of bars on the graph from day 6 and beyond indicates that no VHSV survived incubation at 37°C at those time points in both the dry and wet condition. Stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) was diluted in L15 with 2% FBS to 10^{-2} and was seeded into 80 wells of four 96-wells plate at a total volume of 100 µl per well for each of the dry and wet condition. L15 with 2% FBS was added to 16 control wells of each plate at a total volume of 100 µl per well. All plates were rinsed with D-PBS. After rinsing, nothing was further added to the plates in the dry condition but 100 µl of L15 with 2% FBS was added to the plates in the wet condition. All plates were incubated at 37°C for up to 15 days. At day 1, 6, 10 and 15 time points, 50 000 EPC cells were added to each well of a plate from both conditions. After addition of reporter cells, the plates were incubated at 14°C for 7 to 10 days and then scored with Alamar Blue for reporter cell viability. Death of cells in VHSV exposed wells but not in control wells indicate the presence of active VHSV.

3.5.6 Comparison of VHSV inactivation across all incubation temperatures in dry and wet conditions

The inactivation of VHSV increased as the temperature increased in the dry condition. After 1 day of drying, all plates up to 26°C reported about the same number of wells with infectious VHSV. As the incubation time increases at the respective temperatures, the differences in VSHV inactivation become noticeable by the 96-well plate method (Figure 2-16, Panel A). By the 10th day of incubation, VHSV in the all wells were completely inactivated at 26°C and above. By the 15th day, only VHSV incubated at 14°C and below survived dry (Panel A). VHSV in wells incubated at 14°C and room temperature appear to have more stable linear inactivation rate over time with survival at 14°C decreased by about a quarter at every subsequent time point and survival at room temperature decreased by about one third at every subsequent time point (Panel A). Within the sensitivity of the 96-well plate method, VHSV inactivation was not detectable when incubated in wet condition up to 26°C over the 15 days incubation period; all of these plates show the 80 maximum number of wells with active VHSV (Panel B). At 37°C, the inactivation of VHSV is significant and in stark contrast when compared to the other temperatures. Less than 20 wells contain active VHSV only after 1 day of incubation at 37°C and no well contain infectious VHSV at all by the 6th day (Panel B).

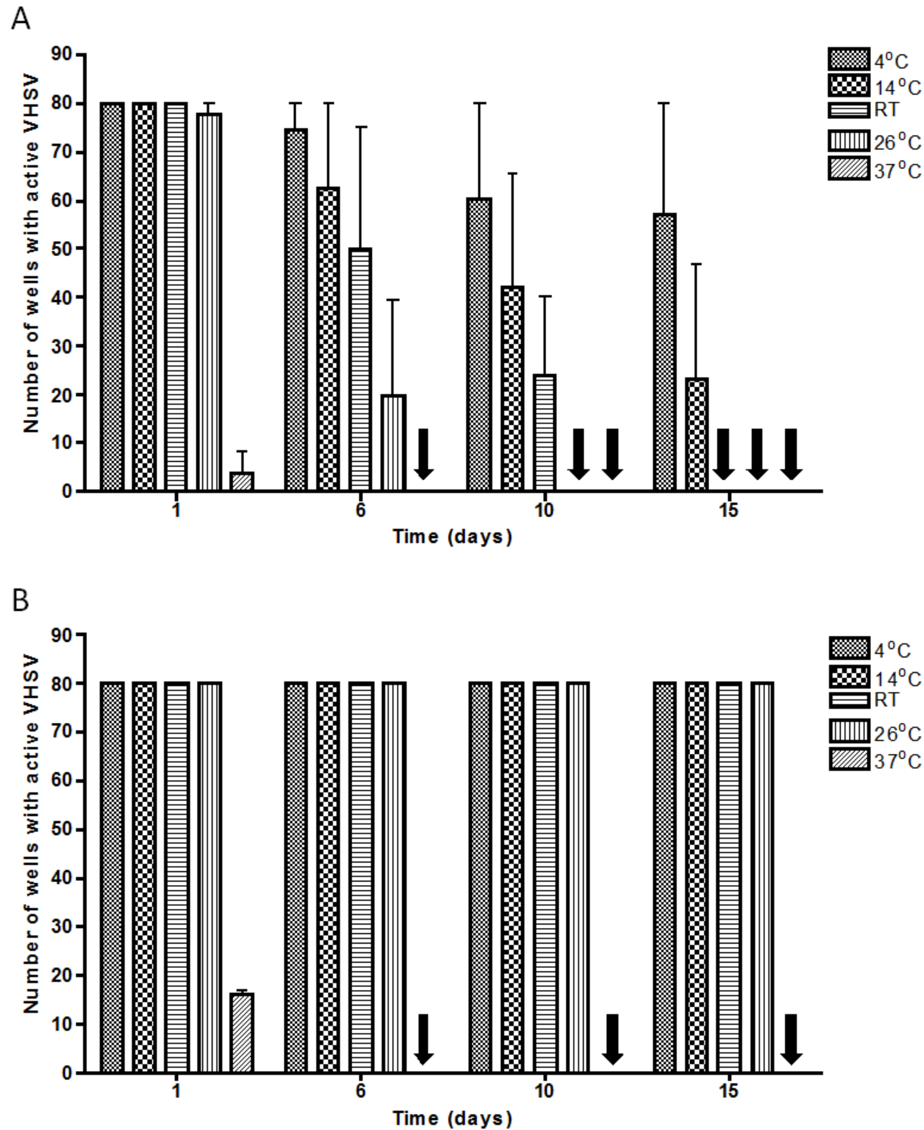

Figure 2- 16. Survival of VHSV in dry and wet conditions on 96-well plates across all temperatures.

The survival of VHSV in dry condition at 4°C, 14°C, room temperature, 26°C and 37°C is compared in Panel A and the survival of VHSV in wet condition at those same temperatures is compared in Panel B. The arrows indicate time points at which there is no VHSV survival at the respective temperature. RT stands for room temperature. Stock VHSV ($10^{8.8}$ $TCID_{50/200\ ul}$) was diluted in L15 with 2% FBS to 10^{-2} and was seeded into 80 wells of four 96-wells plate at a total volume of 100 µl per well for each of the dry and wet condition. L15 with 2% FBS was added to 16 control wells of each plate at a total volume of 100 µl per well. All plates were rinsed with D-PBS. After rinsing, nothing was further added to the plates in the dry condition but 100 µl of L15 with 2% FBS was added to the plates in the wet condition. The plates were incubated at either 4°C, 14°C, room temperature, 26°C or 37°C for up to 15 days. At day 1, 6, 10 and 15 time points, 50 000 EPC cells were added to each well of a plate from both conditions at incubation temperature. After addition of reporter cells, the plates were incubated at 14°C for 7 to 10 days and then scored with alamar Blue for reporter cell viability. Death of cells in VHSV exposed wells but not in control wells indicate the presence of active VHSV.

CHAPTER 4
DISCUSSION

4.1 Rapid scoring of multiwell plates for infectious VHSV using Alamar Blue and Microsoft Excel 2007

Alamar Blue was used in place of the standard procedure of visual inspection in order to rapidly and easily count the number of wells with CPE due to infectious VHSV for the purpose of calculating a $TCID_{50}$. AB is an indicator dye that is reduced by cells undergoing energy metabolism where the magnitude of the reduction can be monitored with a fluorescent plate reader as relative fluorescence units (RFU) (O'brien et al., 2000). As used here in virology for the first time, the method relies on near complete or complete destruction of the reporter cells monolayer by the virus; this maximizes the difference in RFU readings between the control and viral infected wells. Wells with reporter cell monolayer in early stages of infection with mostly viable cells produce high RFU readings that will fail to meet the cut-off criteria and be assigned as a false negative for presence of virus. The long incubation period of 7 to 10 days for VHSV in EPC cells ensures that most or all of the infected monolayer reach total destruction even when the initial titer is low, thereby minimizing false negatives. In contrast, when visually examining infected wells under the microscope for CPE, an incubation period of 4 to 7 days is usually enough to examine even early stages of CPE development. Although the visual examination method requires less incubation time, a viewer trained at recognizing CPE is required and the accuracy of the method is dependent upon the viewer's ability and subjectivity, and can vary between different viewers.

Since the readings from the AB assay is a table of numbers, computer programs, such as Microsoft Excel 2007, can be employed to perform rapid comparisons between RFU values of an individual exposed well with that of the cut-off criteria and automatic scoring of each well for infectious VHSV, positive (1) or negative (0), can also be accomplished at the same time. The use of 1 (instead of "+") and 0 (instead of "-") allows for easy summation of the total number of wells with infectious virus. The process of scoring and summation can be streamlined by creating a scoring template in the computer program from which scoring can be as easily accomplished as copying and pasting the RFU values of each plate into the template. If the setup of the $TCID_{50}$ is modified on the 96-well plate to include more or less dilutions or number of replications per

dilution, then the template can be easily adjusted to match the new setup. Different multiwell plates, other than the 96-well plate, can also be used. This method is not just limited to $TCID_{50}$ calculations but can be adapted to other similar situations such as in the SCT method and the example given in Appendix 3. AB works with both eukaryotic and prokaryotic cells and can also be used for high throughput mutational screening of many different viruses using their host cell line. The advantage of the AB method is that it is significantly less laborious than visual examination when numerous 96-well plates are being scored for presence of infectious virus and there is no need to account for differences in scoring between viewers. One limitation is that this method must be used with an adherent cell line because non-adherent cells will be lost in the washing step. Also, the control wells and virus-exposed wells should be done on the same plate such that both types of wells are read at the same time by the plate reader to ensure that the differences in RFU is not caused by differences in reading time.

4.2 Examining the elution transfer (ET) method of VHSV

4.2.1 The events from attachment of VHSV to appearance of CPE on reporter cells

Two different methods were applied to study the elution of infectious VHSV that was dried for 6 days at room temperature on different surfaces: agitated elution (the chemical/mechanical elution) and static elution (the two-compartment chamber method). For agitated elution, VHSV was seeded and dried on three different types of 60 mm Petri dish surfaces: low cell bind (LB), conventional tissue culture treated (polystyrene dishes), and soda lime glass dishes. In agitated elution, different eluting solutions were used, but at some point the solutions were pipetted, usually vigorously. The eluants were used immediately or frozen at -80°C, in which case the virus must have been able to survive a freeze thaw cycle in order to infect susceptible reporter cells. For static elution (the two-compartment chamber method), VHSV was dried on 6-well cell insert containing a PET track-etched membrane surface. In static elution, absolutely no pipetting or freezing is done and elution occurs in the same culture system as the reporter cells. For this reason the eluting solution must be compatible with cells which in this case was L-15 with 2 % FBS. Regardless of the surface types, infectious VHSV must undergo the following sequence of events in order to be detected in reporter cells. The first event is the attachment and retention of VHSV to the surface; VHSV particles must first bind to the surface and remain attached after the surface is gently washed. The attached virus must then survive the process of desiccation for 6 days. Afterward, VHSV must then be detached intact by the elution solution from the surface and be infective.

4.2.2 The attachment of VHSV to surfaces

In the first event, the initial attachment and retention of VHSV will be dependent upon the types of surface used. Although the attachment aspect was not directly studied in the two ET methods, the fact that infectious VHSV was eluted from and shown to remain infectious on some of the LB and polystyrene dishes indicates that VHSV did attach to those dishes. However, the fact that infectious VHSV was not eluted from any of the glass dishes and was also not infectious in the glass dishes when reporter cells were added (Table 3) suggests one of the following scenarios. VHSV either did not initially attach to the glass surface or attached but did not remain after the initial wash. However, this scenario is unlikely as results from SCT section showed that VHSV did attach to glass and remained infectious after 1 day of drying when reporter cells were added to the glass. Even though SCT did show attachment, how much virus attached is not known. Attempts should have been made, immediately after the initial wash step (day 0 of drying), to quantify how much of the initial seeded amount of virus remained using ET and $TCID_{50}$. VHSV is a virus that is encapsulated in a lipid membrane with protruding glycoprotein spikes on the surface of the envelope. The attachment of VHSV to a surface can occur through one or a combination of the following processes. First, the ectodomain of the viral glycoprotein can directly bind to the surface. Second, FBS proteins in solution can act as a link between the virus and the surface by binding to both. The coating of plastic or glass surfaces with protein is a common method used to prepare substrate suitable for cell adhesion studies (Mrksich, 1998); poly-L-Lysine is commonly used to coat polystyrene plates or glass slides to enhance cell attachment. Third, the virus can attach to the surfaces through the outer hydrophilic layer of the envelope lipid membrane. The tissue culture treated polystyrene plastic dishes from Corning Incorporated has a hydrophilic surface that is negative charged. Treatment of polystyrene surfaces that generate surface hydroxyl groups is important for cell attachment and suggests a possible role of hydrogen bonding in cell attachment (Curtis et al., 1983). The hydrophilic surface could interact with the hydrophilic layer of the viral envelope. The cell culture insert PET membrane surface is also tissue culture treated and could interact with the virus in a similar way to the tissue culture treated polystyrene dishes. One alternative way in which VHSV can remain on the dishes without needing to attach to the surface could be due to left over fluid from aspiration. Although aspiration was done as thoroughly as possible to remove the wash solution, there was always a thin film of liquid remaining on the surface of the dishes that could contain VHSV. This could possibly be the reason why VHSV remained on LB dishes and was most easily eluted from the LB dishes by four out of five elution solution used. The surface of the LB dishes is coated with 2-

methacryloyloxyethyl phosphorylcholine (MPC) polymers which make the surface neutral and inert (Iwasaki and Ishihara, 2005) thereby, reducing both cell adhesion (Koike et al., 2005) and protein absorption (Ishihara and Iwasaki, 1998). The virus that remained, but not necessarily attached, and survived dried on the LB dishes after initial washing could easily be mechanically eluted off with elution solutions, whereas with the polystyrene and glass dishes dislodgment is much more difficult due to the actual attachment of the virus to those surfaces.

4.2.3 The survival and inactivation of VHSV dried on various surfaces

After the retention of VHSV on the various surfaces, the virus must be able to survive dry at room temperature for 6 days. VHSV can be inactivated through the irreversible disruption of the glycoprotein and/or of the envelope membrane. Adsorption of protein to surfaces causes a change in protein conformation that can lead to denaturation if the change is significant (Sadana, 1992). The more hydrophobic the surface, the greater the change in adsorbed protein conformation and the less stable the protein (Steadman et al., 1992). The tissue culture treated polystyrene dish surface is negatively charged and can reduce conformational changes in the viral glycoprotein and so maintains protein stability. If VHSV was to bind FBS proteins and remain attached on surfaces indirectly through the binding of FBS proteins to the surface, then the FBS proteins would undergo conformational change during adsorption and protect VHSV glycoprotein from such changes. Virus that remains unattached on LB dishes after initial washing will have unbound glycoprotein that is protected from conformational changes associated with attachment. These factors can reduce inactivation of VHSV leading to the survival of VHSV on plastic and LB dishes. When lipid membranes are dehydrated, the phospholipids polar head groups are more densely packed leading the lipid membrane to transition from a normal liquid crystalline phase to a gel phase; this transition causes irreversible damage to the lipid membrane (Crowe et al., 1984). The envelope of VHSV could enter a gel phase when dried, potentially leading to inactivation of the virus. The disaccharide sugar, trehalose, is known to preserve membrane integrity during desiccation (Crowe et al., 1984) by mimicking water interaction with membrane to prevent transition to the gel phase (Crowe and Clegg, 1973). VHSV can be dried in the presence of trehalose to determine the role of envelope degradation in viral inactivation.

4.2.4 The role of various solutions in the elution of dried VHSV from various surfaces

Tissue culture water was ineffective at eluting virus from any of the dish surfaces. If the virus binds to the surfaces through hydrophobic interaction, as might be possible in the case of

the low cell bind and glass dishes, then addition of pure water will only enhance the retention of virus to the surface. However, in the case of the negatively charged polystyrene dishes, even though water is polar, it might not be able to overcome the electrostatic interaction between the virus and the charged surface. Reference freshwater contains high concentrations of calcium, chloride, magnesium, carbonate and sulfate ions. These are antichaotropic ions which promote hydrophobic interaction by stabilizing the ordering of water molecule; hydrophobic groups cannot interact with the increased ordering of water so they interact with other hydrophobic groups (Gerba, 1984). Antichaotropic salts could promote viral elution from the negative charged polystyrene dish if electrostatic interaction was the only force at play. However, they must be strong enough to overcome the interaction between the virus and the dish. This might not be the case as these are ions with small charge. Reference freshwater was not able to elute virus from polystyrene dishes. The five most highly concentrated ions in seawater are chloride, sodium, sulfate, magnesium and calcium ions. These are antichaotropic ions similar to those in the reference freshwater except are present at a much higher concentration than those in the reference freshwater; they can also promote hydrophobic interaction. Chloride and sulfate ions are the first and third most highly concentrated ions in seawater respectively. They are both negatively charged and at a high concentration, they can compete with the negative charged polystyrene surface to bind the virus and promote elution. Increasing concentration of cations has been shown to reduce viral adsorption to positively charged filter (Hou et al., 1980). Therefore, presence of a high concentration of ions of the same polarity as the surface can promote viral elution. The composition of L15 with either 2% FBS or 10% FBS is a mixture of: 1) antichaotropic ions such as magnesium, calcium, potassium, sodium, chloride, and sulfate ions, 2) polar amino acids such as arginine, lysine, asparagines, and glutamine, 3) hydrophobic amino acids such as valine, leucine, isoleucine, methionine, phenylalanine, tryptophan and cysteine, and 4) FBS which is uncharacterized serum. The fact that both L15 with either 2% FBS or 10% FBS were consistently able to elute VHSV from different plastic surfaces, indicates that both electrostatic and hydrophobic interaction may play a role in virus absorption. The antichatropic ions in L15 could disrupt electrostatic interactions while the hydrophobic amino acids could disrupt hydrophobic interactions between the virus and surfaces; the proteins and lipids present in FBS can also aid in disrupting these interactions leading to the displacement of the virus from the surface. The combination of electrostatic and hydrophobic disruption possibilities separates L15 with 2% FBS or 10% FBS from the other elution solutions which do not contain both possibilities. There seem to be no noticeable differences in elution between 2% FBS and 10% FBS.

4.2.5 The potential inactivation of VHSV during and after elution

Even if some VHSV survived drying, the reason for the very low untitratable amount could have been inactivation by the mechanical action (pipette mixing) of elution or by the freeze/thaw process. Pipette mixing of the elution solution in an attempt to dislodge any potentially bound virus could have further disrupted the structural integrity of the viral envelope that is already weakened by desiccation. Pipette mixing also increases the exposure of VHSV to the air-liquid interface. Trouwborst et al (1974) showed that bacteriophage T_1 and the Semliki Forest animal virus were inactivated by exposure to liquid/air interfaces generated by bubbling of air through solution. Hydrophobic regions of the virus will be absorbed to the air side of the interface resulting in conformational changes (Trouwborst et al., 1974). The binding domain of VHSV glycoprotein to phosphatidylserine on host cells contain stretches of heptad repeats with a hydrophobic amino acid at about every 4th position (Estepa and Coll, 1996). These hydrophobic regions can increase glycoprotein absorption to the air side of the interface that can lead to damaging structural changes. The potentially eluted virus was all frozen at -80°C in the specific elution solution without addition of any cryopreservant to the elution solutions. This can result in further inactivation of eluted virus if formation of ice crystals exerted pressure on and disrupted the viral envelop. Sardines that were commercially blast-frozen after death due to infection with VHSV showed a 1000 fold or 99.9% reduction in viral titer in the kidney and spleen (Arkush et al., 2006). In the sardine case, VHSV was frozen inside fish tissue which could have had some protective effect against freeze/thaw inactivation. For eluates that were frozen with the tissue culture water, reference freshwater and seawater, the only protein in those solutions would be from co-elution of dried FBS protein along with the virus. However, the lack of additional fresh proteins in those solutions could have led to more severe viral inactivation than in the case of L15 with either 2% FBS or 10% FBS. Dishes, such as the low cell bind and tissue culture treated polystyrene, that was eluted with L15 with either 2% FBS or 10% FBS were more consistent at producing infectious virus.

In summary, the resulting low recovery of dried virus from all dish types could be due to the synergy of all these deleterious effects: desiccation, mechanical disruption, and freeze/thaw inactivation. One way to eliminate the potential freeze/thaw inactivation effect is to repeat the elution experiment and do the titration without freezing the eluent.

4.3 Examining the object associated transfer (OAT) method of VHSV

4.3.1 Wet Object-associated transfer of VHSV

All wet objects were able to transfer infectious VHSV. These objects were glass beads, fishing hooks, and pieces of fishing lines, pop cans, and plastic water bottles. All objects were incubated with the virus in L-15 with 2% FBS, rinsed in buffered saline, and then kept wet in L-15 with 2 % FBS for up to 10 days before being transferred to reporter cell cultures where CPE developed in the cell monolayer. The objects could have transferred VHSV by two different routes, working separately or perhaps together at differing degrees. Firstly some virions could have attached to the surfaces of these objects and remained attached during the rinsing and subsequent wet incubation but came off during the time in the reporter culture to cause an infection. The viruses would likely have to come off the surfaces to cause an infection because direct contact between the objects and the monolayers was slight or nonexistent. For example, the fishing line pieces tended to float in the medium above the cells. As for the second transfer route, some virions could be in the small volume of rinsing buffer that remained after the buffer was removed. Even though the wash buffer was removed by aspiration, the container still remained damp. The traces of wash buffer possibly containing some virus would then be mixed with the wetting solution. During the transfer of the object to reporter cells, small volume of wetting medium would associate with the object as a thin film of liquid and also be transferred into the reporter culture. The volume of medium carried over with an object at each transfer step would vary with the object size and shape, but can be roughly estimated at 100 µl. With this value, the fluid associated with the object will have been diluted approximately 200, 000 when the fluid mixes into the reporter culture medium. Regardless of the transfer route, the experimental conditions likely maximized the detection of virus transfer by wet objects. The 2 % FBS in L-15 would be expected to enhance VHSV survival, whether the viruses were attached to the object or in solution. This is because FBS has been shown to extend the survival in aqueous media of several fish viruses, including VHSV (Kocan et al., 2001). Therefore the experimental protocol evaluated the capacity of wet objects to transfer VHSV under the most optimal conditions and not those most likely to be found in the environment. Despite this, a clear difference was seen in the ability of wet objects to transfer VHSV.

Unlike the other objects, which transferred the virus after being kept wet for at least 10 days, the fishing hooks had lost this ability by 10 days. Several mechanisms likely contributed to this. The surfaces of the fishing hooks might have supported the attachment of fewer viruses, been

more potent at inactivating attached viruses, and/or retained the virus more tightly. However an important feature of fishing hooks that likely contributed to viral inactivation was rusting. The longer the fishing hooks had been kept wet, the quicker did rust from them appear in reporter cultures. For a hook that had been kept wet for only 1 day, rust took several days to develop in the reporter culture, which did develop CPE. Whereas for a hook that had been kept wet for 10 days, the rust was evident even before transfer of a fishing hook to the reporter culture, which did not develop CPE. The iron oxides generated during rusting could have inactivated the viruses even before the hooks were added to the reporter culture. Additionally, in reporter cultures, the cells closest to rusting hooks appeared to be dying or dead, which would prevent the development and spread of an infection in the monolayer. Finally the Danielson hooks rusted quicker than the Mustad hooks, and when wet, Danielson hooks could transfer VHSV only for one day. By contrast aluminum pop can pieces did not rust and continued to transfer the virus for at least ten days. Therefore the ability of metal objects to resist rusting might be critical in determining their capacity to transfer viruses when wet. The survival of viruses on wet metal objects appears not to have been investigated previously. In sports fishing, hooks are often left lodged within fish as a result of the fishing line breaking. With time inside the fish, these hooks rust (Doi et al., 2005), which would have the advantage of impairing their potential to act as fomites.

4.3.2 Dry object-associated transfer of VHSV

Dry stainless steel fishing hooks had no capacity to transfer VHSV, but after one day of drying, pieces of aluminum pop can still did. As suggested above for wet hooks, the virus would have been on these objects as a result of either adhering or being dried onto the object surfaces or both. The drying would have been in the small volume of buffered saline that would have been left on the objects after rinsing. A variety of other viruses have been reported to survive dry on stainless steel and aluminum surfaces for at least a day (Weber and Stiliankis, 2008). The one experimental difference between these reports and the current one is that the virus was dried onto the surface, whereas for experiments in this report, VHSV was allowed to attach and then dried after rinsing. Thus the failure of dry hooks to transfer the virus might be because fewer viruses initially attached to the surface of hooks. This could have been because the surface area of the pop can pieces was simply larger and/or because of differences in the surfaces of the two objects. The hooks have proprietary coatings with the coating of the Mustad hooks being described as 'gold' and the surface coatings of hooks would be expected to differ from those of the pop can pieces and to be more homogenous. The pop can pieces had three types of surfaces: the inner surface of the can, which would have been coated with epoxy resin, the outer surface, which had

paint for the brand name, and the broken edges, which had small surface areas but still might support attachment. The survival of VHSV on these fragments for a day is consistent with the reports of several medically important viruses surviving dry on aluminum for at least a day. This includes Hepatitis A virus, enteric adenovirus, human rotovirus, and coronavirus (Abad et al., 1994; Sizun et al., 2000), although these reports did not state whether the aluminum had any kind of coating or not.

Alternatively the same amount of virus might have attached but viral inactivation by drying was more effective on hooks. Drying is well known to inactivate viruses and to be influenced by several physical factors as well as the type of virus (Mbithi et al., 1991; Songer, 1966; Buckland and Tyrrell, 1962). Differences in object shapes could have affected the drying or evaporation rate and thus the viral inactivation rate. The pop can pieces lay flat in a container during drying, with some buffer fluid being trapped underneath them. Drying on this 'bottom' surface of the pop can pieces was slower than on the 'top' surface. By contrast, drying of the hook was faster and uniform. Additionally, if hooks adsorbed less protein from the virus exposure medium (2 % FBS in L-15) than the pop can pieces, viruses on hooks might have had less protection. For example, HIV on a dry glass surface was inactivated more rapidly when the level of protein was low (Vanbueren et al., 1994). Rusting could have contributed to the VHSV inactivation on dry hooks, but likely was less important than with wet hooks. With dry hooks, rusting did not become visible until after several days in the reporter cultures.

Finally the same amount of infectious virus might have been on the two metal objects, but the viruses were more rapidly released from the pop can pieces by the 2 % FBS/L-15 medium of the reporter cultures. This possibility is difficult to distinguish experimentally from differential inactivation of VHSV on the object surfaces, but is mentioned in order to complete the logic.

Glass beads were able to transfer VHVS after being dry for one day. Survival of the virus on glass is consistent with work on other types of viruses. Several viruses of importance to human and veterinary medicine have been shown to survive on glass, usually glass slides. Edward (1941) first demonstrated this with influenza virus. The virus survived dry on glass slides as long as five weeks, although at this time only 0.01% of the original amount was present. Buckland and Tyrrell (1962) spotted suspensions of several different viruses on glass slides, rinsed them after only 2.5 hours of drying, eluted them with suspension medium, and titrated the medium for virus. Influenza, poliovirus, Coxsackie virus, Rhinovirus B633, Adenoviruses of several types, Reovirus type 2m, and Semiliki Forest virus type 2 all survived. The survival of HIV was studied by placing dilutions of virus onto 13 mm diameter glass coverslips, drying them for 2.5 hours and

placing them into 24-well plates (van Burren et al., 1994). After being left dry in a well for 1, 4, 5, 6 and 7 days, the well received T-lymphoid MT-4 cells, which grow in suspension. The cultures were monitored for CPE, and when initial titer was high, HIV survived for at least 7 days dry. Avian reovirus was dried on pieces of glass windowpane, and at various times afterwards, the surface was sample with a cotton swab (Savage and Jones, 2003). Depending on the virus strain and whether drying had occurred together with fecal matter, avian reovirus survived dry for 2 to 7 days.

Fishing line was able to transfer VHSV after being dry for one day. This was clear for braided line but was inconsistently observed with the monofilament line. Braided line is often made of ultra high molecular weight polyethylene. Monofilament line can be made from a variety of materials. These include nylon, polyethylene, polyethylene terephthalate, polyvinylidene fluoride, and ultra high molecular weight polyethylene. The material of the braided and monofilament lines used in this study was unavailable. Avian influenza virus has been shown to survive dry on polyester (Tiwari et al., 2006), which is polyethylene terephthalate. Many objects, including boats, are made of the synthetic polymers that make up fishing lines. Thus VHSV might survive dry for at least a day on a wide range of objects.

One object is plastic water bottles. Most water bottles are made of polyethylene terephthalate. Pieces from a plastic water bottle were able to transfer VHVS after being dry for one day. Several viruses have been reported to survive dry on plastic objects, but the kinds of objects and the types of plastics from which they were made has been variable and sometimes not described beyond being plastic. Influenza virus survived for approximately two days on the flat bottom of a plastic dishpan (Bean et al., 1982). Survival of several human enteric viruses has been studied on polystyrene, which presumably was tissue culture grade (Abad et al., 1994). Suspensions of virus in a volume of 100 μl were inoculated onto polystyrene wells, dried under different conditions, and sampled after various drying periods. Viruses were eluted by incubating the well with 900 μl of 3 % beef extract in saline for 10 min and then vigorously pipetting 20 times. The eluates were stored at -75 °C before being assayed. Hepatitis A virus, human rotovirus, enteric adenovirus, and poliovirus were able to survive a day or more. Parainfluenza virus appeared more sensitive to drying on plastic than these viruses. The recovery of this virus from laminated plastic was done with MEM at various times after drying (Brady et al., 1990). The virus survived only for 2 hours. Other viruses to be studied include Hendra virus (HeV) and Nipah virus (NiV), which are viruses of emerging concern belonging to the genus *Henipavirus* (Fogarty et al., 2008). These viruses were spread onto polystyrene dishes in a volume of 10 μl

per dish and then air dried for 15 min at room temperature, after which they were left dry at either 22 °C or 37 °C for several hours before the surface was eluted with PBS. The viruses survived for less than 15 min at 37 °C and less than 2 hours at 22 °C. For avian metapneumovirus (AMPV), one strain (reovirus 2) survived dry for at least two days on pieces of polyethylene bag, whereas another strain (S1133) could not be recovered in amounts to be titratable (Savage and Jones, 2003). Both strains survived dry for at least one day on vinyl gloves. Another study found that AMPV and avian influenza virus (AIV) survived dry on plastic and polyester for up to 72 hours (Tiwari et al., 2006).

4.4 Examining the surface-to-cell transfer (SCT) method of VHSV

4.4.1 Mechanism of surface-to-cell transfer of viruses

This appears to be the first description of cells being infected by a surface-to-cell transfer of viruses. However, a variation of the phenomenon might have been going on ever since the first cell cultures in plastic and glass Petri dishes were dosed with viruses over 60 years ago. A small portion of the viruses would likely have attached to the Petri dish surface, and from this site, infected cells. However, infection by this route would have been either unconsidered or considered trivial because the way cultures are conventionally dosed with virus would greatly favour infection from viruses suspended in the medium. Usually cultures are dosed with viruses after cells have been allowed to attach and spread on the Petri dish surface. The viruses would then first come in contact with high affinity receptors on the cells that would allow infection to be initiated before reaching low affinity binding sites on the Petri dish surface. In this thesis, SCT of virus was demonstrated by allowing the viruses to attach first to the Petri dish surface, rinsing the surface, and then letting the cells attach and spread on the virus-coated surface.

Infection from inanimate plastic and glass surfaces with attached viruses could be achieved in several general ways. The first is that viruses could be eluted from surfaces by only the culture medium (2% FBS/L-15) and infect cells from the medium. An alternative way is for the cells to actively elute viruses into the culture medium from where they infect the cells. In the third way infection could be achieved without viruses ever being detached and released into the medium. These mechanisms are discussed below as occurring separately but all three might be operating to varying degrees at any one time.

The 2 % FBS in the culture medium could be eluting viruses from glass and plastic surfaces, whether flat as in Petri dishes or in the form of beads, and eluted viruses could then infect cells.

As discussed previously many proteins and lipids of FBS are able to bind tissue culture surfaces, and in doing so, they might simply displace bound viruses. Displacement could begin as soon as the cells are added to the culture and before the cells have had a chance to attach and spread on the surface. In fact, the cells might be in the act of being infected as they are settling on the surface of the Petri dishes. The reason that 2 % FBS/L-15 alone was rather poor at eluting infectious viruses from surfaces might have been due to the additional pipetting that was done in an attempt to enhance elution by physically dislodging viruses. As discussed previously, pipetting can generate exposure of the viruses to air-liquid interfaces that can inactivate viruses. Therefore adding the cells together with the elution medium (2 % FBS/L-15) is a much more sensitive method of detecting viruses on surfaces.

A test of whether 2 % FBS/L-15 could elute infectious VHSV from surfaces without any possible direct involvement of cells and without pipetting was made in multiwell plates with culture inserts. Inserts divide an individual well into top and bottom compartments. Aliquots of virus solution were placed onto culture inserts, which after a period were rinsed and allowed to dry for various lengths of time. These inserts were placed into wells that had monolayers of EPC cells on the bottom. At the start, the viruses were on the surface of the insert in the top compartment and the cell monolayer was on the surface of the bottom compartment. The insert has 3 µm pores so the medium in the top compartment is continuous with the medium in the bottom compartment. The insert surface is separated from the bottom compartment surface by column of medium approximately 1 mm in height. With time, CPE developed in the monolayer so infectious VHSV was eluted from the culture insert by 2 % FBS/L-15 and travelled to the bottom of the well. The culture set up was reversed so the virus was on the plastic surface of the bottom compartment and the cells were on the insert in the top compartment. CPE developed in the monolayer in the top compartment. Therefore 2 % FBS/L-15 again eluted infectious VHSV from the plastic surface. However, in this case, the virus moved up through the column of medium and through the 3 µm pore to infect the EPC cells. Overall the results suggest that 2% FBS/L-15 can elute infectious viruses from surfaces without any physical disturbance or manipulation and without direct involvement of the reporter cells.

Active elution by the cells of viruses from inanimate surfaces appears not to have been considered before, but such elution could occur by several means. The cells could release factors that displace viruses from surfaces. These could be proteins and/or lipids. If this was the mechanism, elution would be expected to be slow because the buildup of released factors into the medium would take time. Also elution and thus infection would be favored in monolayer cultures

in small rather than large volumes of medium because the critical concentration of factors necessary to elute viruses could be reached more quickly. This mechanism could work on large or small plastic surfaces. However, a variation of active cellular elution can be envisioned that would only occur on surfaces large enough for cells to attach and fully spread. When animal cells attach and spread on a surface, they make physical contact with the underlying substrate substratum at distinct points known as focal contacts (Adams, 2001). In other areas, narrow spaces exist between the substrate and the bottom of the spread cells. In these spaces, factors released by cells could quickly build up and act on the substrate to release viruses, which would be trapped in these spaces, favouring infection of the cells.

Viruses on inanimate surfaces could infect attached cells without detachment of the viruses. Viruses often interact with cells through receptors on cell membranes. Several cell membrane components have been identified as part of a receptor complex for VHSV, including fibronectin and phosphatidyl serine (Bearzotti et al., 1999; Nunez et al., 1998). These viral receptors would be expected to be more specific and have a higher affinity for a virus than receptor sites on a plastic surface. The greater affinity of the receptors on the membranes of cells can be envisioned as 'plucking' the viruses off the surface of Petri dishes or plastic beads. This could begin the moment cells make contact with the surface and continue as they attach and spread. In this scenario the viruses are never free in suspension but are just being passed like a baton from an inert surface to a living surface.

4.4.2 Comparing surface-to-cell transfer method with elution transfer method

When the ET method was used to study the dry survival of VHSV, the amount of virus eluted was so little such that the titer could not be determined. This could be due to poor elution or inactivation as described in the elution transfer section. The SCT method was developed to bypass the elution process by directly adding reporter cells on top of the surface containing the virus. With the 96-well SCT method, the true survival of VHSV in dry and wet conditions and across various temperatures can be compared. The advantage of the SCT method over the ET method is that SCT can be used to study the survival of a variety of viruses regardless of the attachment properties of the virus. If attachment is of concern then viruses can be dried on the surface without washing. Different types of viruses can just be added to and dried on the particular surface of interest. With the ET method, the properties of the virus must be taken into account as an optimal elution solution must be separately determined for virus with different properties. As previously discussed, there are many different factors affecting the elution of virus

from a surface. Low $TCID_{50}$ values could be due to poor elution rather than true viral inactivation.

4.4.3 Using SCT method to study the effect of temperature on VHSV survival in wet and dry environments

The survival of VHSV in dry and wet environments was studied using the STC method. The SCT of virus with 96-well plate method was unable to detect differences in VHSV inactivation when the virus was incubated in the wet environment of L15 with 2% FBS for 15 days at all temperatures tested up to 26°C. This means that for temperatures of up to 26°C, enough VHSV remained viable in each well such that each well showed infectious VHSV when assayed with Alamar Blue. There could have been differences in the actual amount of VHSV remaining in each well across the various temperatures up to 26°C but the SCT method was not sensitive enough to detect it since only a certain threshold amount of virus is required to destroy the EPC monolayer of each well. However, as the incubation temperature was raised from 26°C to 37°C, differences in VHSV inactivation was noticeably detected by even only after 1 day of incubation. At 37°C, very few of the initial VHSV exposed wells contain infectious VHSV after 1 day of incubation. Therefore, 37°C is such a highly lethal temperature for VHSV that it can inactivate all VHSV in most of the wells after 1 day of incubation, whereas such was not the case at 26°C even after 15 days on incubation.

Other researchers have studied the survival of VHSV at different temperatures and in different environments. Hawley and Garver (2008) noticed a significant drop in VHSV survival in raw seawater when the incubation temperature was increased from 4°C to 20°C; VHSV survived for up to 12 days at 4°C but for only 1 day at 20°C. The same pattern of inactivation is true for raw freshwater as the survival of VHSV dropped from more than 25 days to about 5 days when the incubation temperature was changed from 4°C to 25°C (Hawley and Garver, 2008). Using the plaque assay technique of viral titration, Hawley and Garver (2008) were able to detect differences in viral inactivation for temperature of 4°C to 25°C as early as about 5 days, but the SCT of virus for 96-well plate method was not able to detect differences in VHSV inactivation between temperatures of 4°C to 26°C even up to 15 days of incubation. Therefore, in terms of studying viral survival in solution, plague assays and $TCID_{50}$ methods are much more sensitive than 96-well plate SCT of virus method.

However, 96-well plate SCT of virus method was initially designed for studying the survival of virus in dry conditions. The sensitivity of this method for dry condition was evaluated when

various dilutions of VHSV was dried on 96-well plates. Up to 10^{-4} dilution of stock VHSV, no differences in VHSV inactivation was detected when those dilutions were dried for 10 days at room temperature. However, there were noticeable differences when the 10^{-5} dilution was dried but only by the 10th day of incubation. It could be that when the virus was diluted to 10^{-5} and dried at room temperature, the starting titer was low enough such that VHSV survival was below the threshold amount for EPC monolayer infection in the well. With the higher titers, inactivation of VHSV by drying at room temperature did not push the amount of VHSV survival to below the threshold amount by the 10th day. Therefore, the initial seeding titer of VHSV does affect the length of time in which the surface can retain infectious virus. This observation is also true for the survival of cytomegalie virus, as lower starting virus lead to less retention on latex surface after washing (Faix, 1987).

The reason 96-well plate SCT of virus method was used for the wet condition is to compare it with the dry condition. In the dry condition, the 96-well SCT of virus method was very effective at detecting differences in VHSV inactivation for temperatures from 4°C up to 26°C by the 6th day of incubation as such was not the case in the wet condition. Therefore, it can be concluded that VHSV is much more sensitive to inactivation in a dry environment than in a wet environment. Generally this has also been the case in studies with viruses infecting mammals (Sizun et al., 2000), but this is the first demonstration of this with a fish virus. One reason for the great difference in VHSV inactivation in dry versus wet environment, as discussed previously, could be that the viral lipid envelop enters the very damaging gel-phase in dry environment, whereas in the wet environment the membrane remains in the fluid-phase due to interaction with water molecule in solution.

The sensitivity of VHSV in dry conditions greatly increases as the incubation temperature increases. This is a unique observation because most other studies on the dry survival of viruses have been done with viruses of importance in human and veterinary medicine and the most significant, and usually the only temperature studied, is room temperature (Brady et al., 1990; Tiwari et al., 2006; Savage and Jones, 2003; Sizun et al., 2000). An exception to this is a study with hepatitis A virus (Mbithi et al., 1991). The effect of temperature on the dry survival of this virus was complex, with both temperature and relative humidity (RH) influencing the outcomes. Hepatitis A survived better at low than at high temperatures, and surprisingly survival was better at low than at high RH.

In the current study, the interplay of temperature, evaporation rate, and RH might be contributing to the more rapid inactivation at high temperatures. The wells at low temperatures

were visibly moist longer than the wells at higher temperatures, suggesting a higher RH and slower evaporation rate. In the case of phages these later conditions favored survival (Ward and Ashley, 1977). High evaporation rates are thought to accelerate exposure of viruses to air at the liquid-air interface, causing inactivation. However, increasing temperature might be inactivating viruses by causing viral proteins to denature more frequently. In the future, these possibilities might be distinguished by allowing the virus to dry on a surface for a fixed period, say one day, and at fixed temperature, say 26 °C. Then the surfaces could be placed at different temperatures and monitored with time for the presence of infectious virus. Although RH might still be hard to keep constant at all temperatures, evaporation would presumably have been completed and the rate of evaporation would have been constant. Therefore this could be eliminated as a variable.

The ability of VHSV to survive dry on inanimate surfaces has implications for the potential capacity of marine debris to have a role in transmitting viruses. Debris that is temporarily beached and dried could still retain some infectious virus. However, as being dried on surfaces appeared to make VHSV more sensitive to temperature inactivation, the virus might be more susceptible to inactivation by other environmental variables. An interesting variable to investigate would be sunlight. The potential of marine debris to transport and transmit viruses is discussed more thoroughly in later sections.

4.5 Possible role of floating debris in transporting fish viruses in the aquatic environment

Several observations in this report suggest that floating debris has the potential to move or transport fish viruses in the aquatic environment. To have such an action, the debris must be capable of supporting the attachment and survival of a virus. This has been demonstrated with several kinds of debris surfaces using and VHSV. At least some infectious VHSV was adherent to or associated with the surfaces of pieces of fishing line, plastic water bottles, and pop cans after the pieces had been rinsed once with saline buffer to remove non-adherent virus, incubated wet for up to 10 days at room temperature, and lifted to other culture vessels. The vessels had EPC cells that reported the presence of infectious VHSV by developing CPE. The conditions used to demonstrate the capacity of these debris surfaces to transfer VHSV would have to be regarded as the most optimal possible and in the environment several factors might restrict such transfer. These are discussed below.

The amount and/or kind of proteins that were present during the adherence step and in the subsequent wet incubation period might have promoted more VHSV adherence and survival on

these surfaces than could any protein in freshwater or seawater. In all the experiments the virus was in L-15 with 2 % FBS during the initial incubation of VHSV with a surface; therefore, serum proteins will be adhering to these surfaces along with the virus. Although most L-15 components would be removed after the surfaces are rinsed with saline buffer, some of the serum proteins will remain and more will attach during the period of wet incubation, which is also done in L-15 with 2 % FBS. Generally serum proteins have been noted to promote the survival of viruses (Kocan et al., 2001). For virus produced and shed from fish, the initial receiving water might have a relatively high protein concentration because viruses would be being released from dead fish or in ovarian fluid. Thus adherence to floating debris could take place in the presence of high protein concentrations. However, subsequently as debris floated away from the viral source, the protein levels in the surrounding water would be expected to drop off dramatically. Some protein is found in the dissolved organic matter (DOM) of seawater and freshwater but the concentrations would be much lower than in serum and the main source of these proteins would be microorganisms rather than vertebrates (Anoue et al., 1995; Ogawa and Tanoue, 2003). Inanimate surfaces can absorb proteins from seawater (Taylor et al., 1994). Whether proteins from microbes would protect VHSV is unknown but is something that could be investigated in the future.

After the adherence of the VHSV to pieces of fishing line, plastic water bottle, and pop can, two subsequent incubation conditions favoured the transfer of infectious virus, but these might not be achieved consistently in natural water bodies, and thus the movement of viruses on these objects might be limited in the environment. Firstly, all experimental incubations were done in the dark, whereas in the environment these objects likely would be exposed daily to sunlight. Sunlight is well known to inactivate bacteriophages (Wommack et al., 1996), and presumably inactivates fish viruses as well. However situations can be envisioned where debris might be in the dark for long periods. Debris in the ballast water of ships or trapped under ice would be shielded from sunlight. Also viruses on the inside surfaces of pop cans would be protected from light. Secondly, all the experimental incubations were static, whereas for floating debris in the environment waves might be expected to agitate the objects, which might repeatedly expose viruses on the surfaces to the air-water interface. Viruses can be inactivated at air-water interfaces. Therefore, wave action might limit the ability of floating debris to carry infectious viruses.

4.6 Possible role of floating debris in transmitting viruses to fish

Although VHSV might survive on the surface of floating debris for at least 10 days, transmission of viruses to fish would likely require close contact between the debris and fish. This is most likely to be achieved with plastic microlitter, which is abundant in the ocean (Thiel et al., 2005). To have such an action, plastic must be capable of supporting the attachment and survival of a virus. This has been demonstrated with VHSV: at least a proportion of the virus remained on plastic surfaces after one rinse with saline buffer and remained infectious at low temperatures for at least two weeks. The literature suggests that close contacts between cells and plastic debris could develop at several sites in fish.

One site is the GI tract. The GI tract is suggested because plastic microliter has been found in the GI tract of marine birds and mammals (Eriksson and Burton, 2003; Petry et al., 2009) and because plastic beads have been found in the GI tract of catfish after experimental exposure to them (Glenney and Petrie-Hanson, 2006). Unless being attached to plastic makes the viruses more susceptible to inactivation, VHSV on plastic litter would be expected to survive in the fish GI tract because this virus has been reported to survive in the more acidic bird GI tract (Peters and Neukirch, 1986). Yet, the fish GI tract is normally not thought to be a site of VHSV infections. However, small plastic litter with bound viruses might aid viral internalization because in rodents and humans plastic particles were translocated from the gut lumen to the inside (Hussain et al., 2001). Once inside, particles of the appropriate size (~0.5 to 5.0 μm in diameter) could be phagocytized.

Other possible sites of close contact are the gills, skin, and fins. These are suggested by experiments in which young fish were immersed in water with fluorescent plastic beads. The beads have been found at the sites of microscopic wounds in skin and fins and adhered to superficial epithelial cells (Kiryu and Wakabayashi, 1999). In other studies fluorescent beads were phagocytized by epithelial cells of skin, nares and gills, as well as by underlying macrophages of skin and gills (Glenney and Petrie-Hanson, 2006; Moore et al., 1998). Beads also were observed in some cases to move to the spleen and kidney (Glenney and Petrie-Hanson, 2006; Moore et al., 1998). Whether plastic debris too large for cells to phagocytize (> 5.0 μm in diameter) can make close contact with gill and skin epithelial cells is unknown, but larger wood debris (< 1.0 mm) has been found lodged between gill lamellae of coho salmon (Magor, 1987). Interestingly the gill often appears to be site the where VHSV infections start (Neukirch, 1994).

Finally to have a role in transmitting viruses to fish, viruses bound to plastic surfaces must be able to infect nearby cells. This was demonstrated with VHSV in two different ways. Firstly, viruses were eluted from plastic surfaces by L-15 with 2% FBS and able to infect cells within approximately 1 mm of the surface. Secondly, plastic tissue culture dishes with attached viruses infected fish cells upon their attachment and spreading on the plastic surface. Thus bringing fish cells and surface bound virus closely together should make infection possible. The most likely way that this could be achieved in oceans is through viruses being bound to plastic microlitter.

This line of reasoning leads to interesting conjecture about other particles in seawater and their possible role in viral disease transmission. Seawater naturally has submicron particles (Wells and Goldberg, 1992). These can range from 0.001 to 1 μm in size and can be divided into three classes based on their composition: organic matter, inorganic colloids, and clay. Whether fish viruses can attach and remain infectious on these kinds of particles is unknown.

Appendix A

A sample $TCID_{50}$ calculation

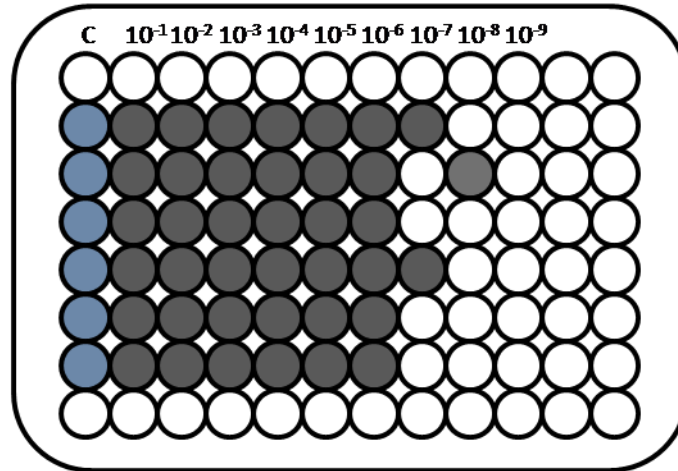

Figure A- 1. Sample $TCID_{50}$ of stock VHSV showing CPE.

Control wells are shown in Blue. Wells positive for CPE as visually identified are shown in black. This plate shown an n=6 for every dilution. The total volume for each well is 200 µL.

The Karber formula for calculating $TCID_{50}$ (Karber, 1931)
Log $TCID_{50}$ = A – D (S – 0.5)

Where,
A is the last dilution index for which all cultures are infected. For the example in Figure A-1. **A = -6** since the 10^{-6} is the last dilution where 6 out of 6 exposed well showed CPE.

D is the log of the dilution factor. For the example in Figure A-1, the dilution factor is 10, therefore, **D = log 10 = 1**.

S is the sum of the ratio of wells with positive CPE out of 6 exposed well starting from the last dilution which all 6 wells are infected to the first dilution were no wells are infected. In the Figure A-1 example, the last dilution which all wells are infected is 10^{-6} and the first dilution which all wells are uninfected is 10^{-9}.
10^{-6} = 6/6 wells infected = 1
10^{-7} = 2/6 wells infected = 0.333
10^{-8} = 1/6 wells infected = 0.167
10^{-9} = 0/6 wells infected = 0
S = (1+0.333+0.167+0) = 1.5

Log $TCID_{50}$ = A – D (S – 0.5)
Log $TCID_{50}$ = -6 – 1 (1.5 – 0.5)
Log $TCID_{50}$ = -7

Virus Titer = 10^7 $TCID_{50/0.2\ ml}$ for the example in Figure A-1

Appendix B

Sample scoring of infectious virus in wells of a 96-well $TCID_{50}$ plate using Alamar Blue (AB)

Alamar Blue solution contains resazurin which can be reduced by fish cells during energy metabolism. The healthier the EPC monolayer the more resazurin that can be reduced leading to a higher RFU when measured with the SpectraMax GEMINI XS plate reader. Monolayer of EPC cells that are either in the process of being destroyed or completely destroyed will produce low RFU readings. Incubation of EPC reporter cells with VHSV at 14°C for the long period of 7 to 10 days ensures VHSV have enough time to nearly completely or completely destroy EPC monolayer and thereby, maximizing the differences in RFU readings between control and exposed wells. Table A-1 shows a sample reading of a 96-well $TCID_{50}$ plate measured with AB. The RFUs produced by the control wells are averaged. The RFU value in each well of each VHSV dilution is compared to the average RFU value of the control wells and if the exposed well is less than half of the control average, then that exposed well is scored as positive for infectious VHSV. In the example in Table A-1, half of the average control wells RFUs is 885.15 units. All wells exposed to VHSV from 10^{-1} to 10^{-8} dilution shows presence of infectious VHSV since their individual RFUs are significantly lower than 885.15 units. However, when VHSV was diluted to 10^{-9}, only 2 out of the 6 exposed well show infectious VHSV. Four wells of the 10^{-9} dilution did not show infectious VHSV as their individual RFUs (yellow cells in Table A-1) are higher than 885.15 units. The total number of wells with infectious VHSV in each dilution can be tallied and used to calculate $TCID_{50}$ values as described in Appendix A.

Table A- 1. 96-well $TCID_{50}$ plate showing relative fluorescent unit (RFU) measured with AB

Control	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
1666.271	130.971	134.456	133.301	130.376	131.541	128.157	131.705	132.708	2133.767
1725.161	131.673	130.022	130.249	124.302	131.607	126.214	134.505	132.705	131.966
1771.409	135.332	131.76	129.443	126.543	131.084	130.63	128.764	137.998	1992.941
1750.183	130.764	135.334	134.905	131.309	134.32	132.64	140.59	136.82	2041.893
1755.464	132.124	132.656	135.921	129.093	136.873	128.264	134.02	131.244	2023.665
1953.749	132.676	137.821	142.939	135.572	135.145	128.892	136.598	134.117	139.703
Total wells with VHSV Per Dilution (n=6)	6	6	6	6	6	6	6	6	2

Average Control RFUs = 1770.37 Half of Average Control RFUs = 885.18

The average control RFUs is calculated by summation of all the RFU values in the control column and dividing by 6. The half of average control RFUs is calculated by taking the average control RFU value and dividing by 2.

Appendix C

Sample scoring of the number of wells with infectious VHSV on a 96-well SCT plate using Alamar Blue (AB)

Alamar Blue solution contains resazurin which can be reduced by fish cells during energy metabolism. The healthier the EPC monolayer the more resazurin that can be reduced leading to a higher RFU when measured with the SpectraMax GEMINI XS plate reader. Monolayer of EPC cells that are either in the process of being destroyed or completely destroyed will produce low RFU readings. Incubation of EPC reporter cells with VHSV at 14°C for the long period of 7 to 10 days ensures VHSV have enough time to nearly completely or completely destroy EPC monolayer and thereby, maximizing the differences in RFU readings between control and exposed wells.

Table A-2 shows the raw AB RFU value for a sample 96-well plate that was exposed with VHSV and dried for 10 days at room temperature. Wells in column 1 and 2 of the plate are controls and were not exposed to VHSV. Wells in column 3 to 12 of the plate were exposed to VHSV. Table A-3 shows the outline of the exact same plate as in Table A-2 except that each exposed well was assigned as positive (1) or negative (0) for infectious VHSV. For this particular plate, after 10 days of drying at room temperature, 55 out of the original 80 VHSV exposed wells showed infectious virus. The protocol for scoring each well in Table A-3 is as follows. The RFU values of all the 16 control wells in Table A-2 was averaged and the cut-off criteria (half of average control RFU) was calculated by taking the average control RFU value and dividing by 2. The RFU value of each of the exposed wells in columns 3 to 12 was compared to the cut-off criteria and if the individual well is less than the cut-off criteria then that well is scored as positive (1) for CPE, otherwise it is scored as negative (0). After all the wells are scored, all the positive (1) wells are summed to give the total number of wells with infectious VHSV out of the 80 original wells. The actual scoring of each well and the final tally can all be automatically done using a computer program such as Microsoft Excel. In this case, the "If" function of Microsoft Excel 2007 was used to assign the positive (1) or negative (0) and the "Sum" function was used to add up all the 1's.

Table A- 2. Raw Alamar Blue RFU values for every wells of a sample 96-well SCT plate.

Controls (16 wells)		**VHSV (80 wells)**									
1	2	3	4	5	6	7	8	9	10	11	12
977.12	1111.709	1032.947	1214.414	1043.557	1187.95	1049.024	1094.361	1116.107	131.48	1176.229	1329.115
1090.988	1031.273	136.31	127.113	127.408	126.198	124.633	141.616	126.58	132.685	213.956	1099.3
1225.349	1028.954	1135.751	1090.314	1232.024	131.528	128.29	134.889	126.726	137.957	133.54	1423.642
1246.047	1049.632	126	129.022	1027.096	137.022	126.212	129.387	130.822	138.649	142.522	1283.874
1404.098	1065.759	129.809	125.18	127.378	132.139	131.274	133.284	140.975	131.786	139.763	1535.438
1233.35	1241.014	193.649	124.813	1219.86	134.084	130.403	137.176	127.784	136.641	134.006	1278.441
1219.449	1016.094	130.626	131.889	169.494	133.732	129.67	137.874	134.311	155.308	1508.372	1176.41
1301.236	1302.257	1151.799	135.594	135.988	523.443	139.285	138.19	139.521	1326.904	1442.807	1198.749

Average Control RFUs = 1159.021 Cut-off criteria (Half of Average Control RFUs) = 448.36

The average control RFUs is calculated by summation of all the RFU values in the control column and dividing by 6. The half of average control RFUs is calculated by taking the average control RFU value and dividing by 2. Control wells are in column 1 and 2. VHSV exposed wells are in column 3 to 12.

Table A- 3. Scores of presence of infectious virus in each well of a sample 96-well SCT plate.

Controls (16 wells)		VHSV (80 wells)									
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
		0	0	0	0	0	0	0	1	0	0
		1	1	1	1	1	1	1	1	1	0
		0	0	0	1	1	1	1	1	1	0
		1	1	0	1	1	1	1	1	1	0
		1	1	1	1	1	1	1	1	1	0
		1	1	0	1	1	1	1	1	1	0
		1	1	1	1	1	1	1	1	0	0
		0	1	1	1	1	1	1	0	0	0

Total number of wells with infectious VHSV = 55

The number 1 in each of the well indicates that the well is positive for infectious VHSV. The number 0 in each well indicate that well is negative for infectious VHSV

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