IMPROVED DIAGNOSIS AND MANAGEMENT OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS IN FISH

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Dedication

This dissertation is dedicated to my father, Brian D. Phelps, for the example of how a life should be lived.

Abstract

Viral hemorrhagic septicemia virus (VHSV) is a highly contagious and pathogenic virus, affecting more than 70 farm raised and wild fish species worldwide. A new viral strain (VHSV-IVb) has proven both virulent and persistent, spreading throughout the Great Lakes of North America and to inland water bodies in the region. As this new biological hazard continues to grow, so too must our understanding of the disease. The focus of this dissertation is to improve diagnostic capacity and management of VHSV-IVb to rapidly respond to outbreaks, prevent further dissemination, and scientifically justify current prevention strategies.

To better understand the geographic distribution of the virus, we used a modified real time reverse transcription-polymerase chain reaction (rRT-PCR) assay for high-throughput testing of fish for VHSV. The assay was shown to be twice as sensitive as the gold standard, virus isolation, and did not cross react with other viruses found in fish. In addition, the diagnostic turnaround time was reduced from 28-30 days for virus isolation to 2-4 days for rRT-PCR. To demonstrate the usefulness of the rRT-PCR assay, 115 high priority water bodies in Minnesota were tested by both methods from April 2010 – June 2011. All survey sites tested negative for VHSV by both methods. The survey results have informed fisheries managers on the absence of VHSV in Minnesota and have better prepared them for the eventual arrival of the disease. In addition, the results demonstrate the value of this rRT-PCR as a surveillance tool to rapidly identify an outbreak so that it can be controlled in a timely manner.

The aforementioned rRT-PCR assay (Phelps et al. 2012) along with another (Jonstrup et al. 2012), were evaluated for the potential for sample-induced inhibition from common diagnostic samples, including kidney/spleen, entire viscera, and ovarian fluid. The detection of high, medium, and low VHSV-IVb dilutions in each tissue type was not affected using the assay by Jonstrup et al (2012). However, using the assay by Phelps et al. (2012), the detection of VHSV-IVb was decreased for the kidney/spleen samples spiked with low virus levels and increased for the ovarian fluid spiked with medium virus levels. Entire viscera, the tissue type most likely to inhibit the rRT-PCR reaction, did not affect the sensitivity of virus detection for either assay.

The emergence of VHSV-IVb in the Great Lakes region has resulted in unprecedented regulatory response to better manage the disease. In Minnesota, all VHSV-susceptible fish must be inspected annually prior to intra or interstate movement – a significant bottleneck for the aquaculture industry. In 2009, Minnesota enacted legislation requiring fish for regulatory health inspections to be collected by a Minnesota Department of Natural Resources (MNDNR) approved individual. The so-called Fish Health Collector could be 1) an American Fisheries Society – Fish Health Section Fish Health Inspector or Pathologist, 2) an accredited veterinarian with approved training, or 3) an individual (i.e. MNDNR field biologist) with approved training. In response, a fish health collector training workshop was developed for veterinarians and field biologists to fulfill the MNDNR requirements. A manual was developed to supplement a full-day workshop and provide the basic information and references to perform a fish health collection. This training resulted in a sufficient number of fish health collectors, well

distributed across Minnesota, now available to rapidly respond to a disease outbreak and better serve the regulatory needs of the aquaculture industry.

In the USA, current state and federal fish health regulations target the spread of VHSV-IVb through movement restrictions of live fish but largely ignore the potential for the virus to be spread through the commercial distribution and use of frozen baitfish from VHSV-IVb positive regions. Some state laws do require treatment of frozen baitfish to inactivate VHSV and additional methods have been proposed, but there have been few scientific studies examining the efficacy of these treatments. In an effort to evaluate these treatments, bluegills were challenged with VHSV-IVb, frozen to represent standard industry methods, disinfected by various treatments, and tested for infectious VHSV-IVb using virus isolation. The virus was isolated from 70% of fish subjected to three freeze thaw cycles. All other treatment methods were effective in inactivating the virus, including treatment with isopropyl alcohol, mineral oil, salt with borax, and dehydration. Dehydration followed by rehydration is rapid and effective, and therefore, seems to be the best option for inactivating VHSV-IVb present in frozen baitfish while maintaining their usefulness as bait.

Monitoring or regulating all risk factors for the transmission of VHSV is an infeasible task. A semi-quantitative risk assessment model was utilized to focus VHSV management efforts in Minnesota. The risk of VHSV introduction to major watersheds in Minnesota was directly correlated with proximity to Lake Superior, the only VHSV-positive waterbody in the state. Although the current regulations are uniform across Minnesota, the risk varied for specific locations within the watersheds. For example, the

introduction of game fish for stock enhancement (a common fisheries management practice) was found to be a significant risk factor for VHSV introduction into public waterbodies and waterbodies frequently used for wild baitfish harvest. Aquaculture facilities with strict biosecurity programs and frequent health inspections received the lowest risk scores and were largely considered protected and of low risk for VHSV introduction. These results suggest the current management strategy, based on political boundaries, should be reevaluated. A risk-based management strategy would better allocate efforts to watersheds or specific waterbodies at higher risk and relax efforts in areas of lower risk of VHSV introduction in Minnesota.

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SECTION A: INTRODUCTION

Emerging fish diseases are an important factor threatening already at risk wild fish populations and limiting the growth of Minnesota's private aquaculture industry. This has resulted in increased regulatory and management action by state (MN DNR) and federal (USDA-APHIS) agencies, a significant drain on government and commercial resources and a limiting factor for fish production. One emerging disease of importance is viral hemorrhagic septicemia (VHS), caused by the viral hemorrhagic septicemia virus (VHSV) which induces hemorrhaging in the host and eventual organ failure. Recently a new strain, VHSV-IVb, has spread through the Great Lakes region, causing numerous mass mortality events and loss of millions of fish. All the Great Lake states currently have waters positive for VHSV-IVb, including Lake Superior in Minnesota. Given the history of the disease, it is anticipated that the virus will continue to spread, likely to inland waters of Minnesota in the future. Most of the known susceptible species (n = 28), including walleye, muskellunge, yellow perch, rainbow trout, emerald shiners, and spottail shiners, are ecologically and economically important to Minnesota.

Proactively preparing Minnesota for the eventual arrival of VHSV is critical to limit the impact of the disease on Minnesota's fish populations. At risk is a recreational fishing industry (tourism, sales, production, etc.) valued in excess of $4.3 billion annually that relies on a fishery that is perceived around the world as pristine and healthy. A smaller but substantive at-risk industry is Minnesota's aquaculture industry, valued at over $8.5 million (2005 farm gate sales).

The regulatory response to VHSV has significantly affected the aquaculture industry. The current regulations imposed at the state and federal levels require an annual VHSV inspection on all lots (same species, age, water source) of VHSV-susceptible fish moving within and out of Minnesota. Two major bottlenecks exist with this system. First, there are few certified fish health collectors in Minnesota trying to serve over 400 producers/harvesters. As a result, the costs of the site visit and resulting diagnostic tests are not economically feasible for producers – limiting the movement of fish. The second bottleneck is the time to perform the diagnostic test. The current gold standard is virus isolation in cell culture, which has a 28-day turnaround period. To reduce turnaround time and rapidly respond to a VHSV outbreak, a real-time reverse transcriptase PCR (rRT-PCR) assay is needed. Several assays have been developed; however, they lack desired specificity and speed for practical use in Minnesota.

Management strategies for VHSV vary across state jurisdictions; however, the majority focus their efforts based on political boundaries, not actual risk. This highly inefficient approach considers the VHSV risk equivalent within a state or region regardless of proximity to known VHSV-positive waters or site specific intervention strategies. Reevaluating the current strategy based on a scientifically justified risk assessment is needed. A risk-based management strategy would better allocate efforts and limited resources to watersheds or specific waterbodies at higher risk. Easing regulation in areas or on farms of lower risk of VHSV introduction would in turn strengthen the fish production industry in Minnesota with minimal increased risk of VHSV introduction.

Overall project goal

To better prepare Minnesota for the introduction of viral hemorrhagic septicemia.

Specific objectives

1. **Diagnostics:** Develop and optimize an rRT-PCR assay for the detection of VHSV in fish
2. **Surveillance:** Perform survey of Minnesota's aquaculture industry and high use recreational waters for VHSV
3. **Training:** Develop and offer a training program to certify fish health collectors in Minnesota
4. **Baitfish treatment:** Identify treatment options to inactivate VHSV-IVb in frozen baitfish
5. **Risk assessment:** Conduct an introduction risk analysis for VHSV in Minnesota waters

SECTION B: LITERATURE REVIEW

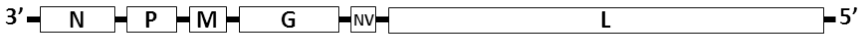
B.1 Characterization of viral hemorrhagic septicemia virus

Viruses belonging to the order *Mononegavirales*, family *rhabdoviridae*, are an important pathogenic group, infecting a broad host range within the plant and animal kingdoms, and include notable diseases such as rabies, vesicular stomatitis, infectious hematopoietic necrosis, and viral hemorrhagic septicemia (VHS) (OIE 2009). The latter is caused by the viral hemorrhagic septicemia virus (VHSV) and more specifically grouped within the genus *Novirhabdovirus*.

Morphologically, VHSV is characterized by a distinct bullet shaped viral particle, approximately 70 nm in width by 180 nm in length. The envelope of the virion is covered with small glycoprotein projections, which are important for virus adsorption and attachment. As with most enveloped viruses, VHSV is relatively unstable outside the host (Smail 1999).

The VHSV is composed of a non-segmented, negative sense, single stranded RNA genome. The genome is approximately 11,000 bp in length, which consists of six open reading frames, including the nucleoprotein (N), phosphoprotien (P), matrix protein (M), glycoprotein (G), nonstructural viral protein (NV), and the polymerase (L) (Figure B1) (Schutze et al. 1999, Betts and Stone 2000, Ammayappan and Vakharia 2009) (Figure B.1).

Figure B.1. Organization of viral hemorrhagic septicemia virus genome, consisting of six open reading frames: N, P, M, G, NV, and L.



Following infection, the six genes are progressively transcribed from the 3' to 5' end. Given that the N-gene resides at the 3' end, it is consequently the most abundant gene and thus the target for many diagnostic assays (Garver et al. 2011). The G-gene, encoding for the surface antigen, is the primary target for vaccines (Lorenzen et al. 1998, Lorenzen et al. 1999) and phylogenetic analysis (Einer-Jensen et al. 2004, Thompson et al. 2011).

B.2 Origin and evolution

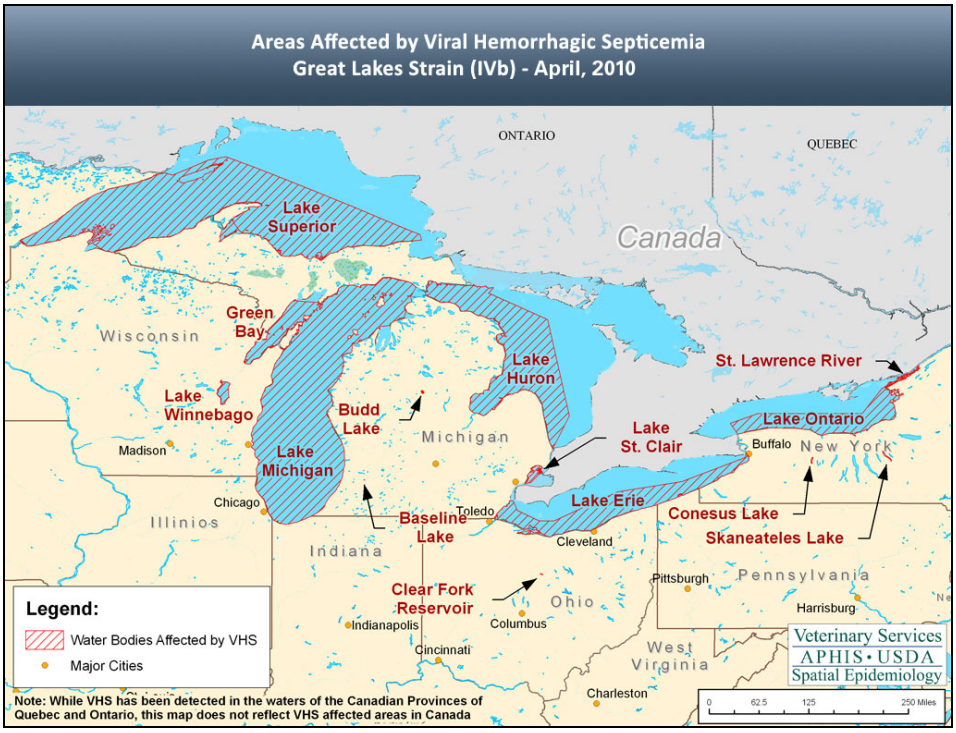
The first reports of viral hemorrhagic septicemia-like symptoms, including kidney swelling and liver degeneration, were documented by Schaeperclause (1938) in Danish rainbow trout farms. Similar conditions were subsequently observed in Poland (Pliszka 1946), Denmark (Schaeperclause 1954, Rasmussen 1965), France (Besse 1955, Bellet 1965), and Italy (Ghittino 1965). Although suspected, a viral etiology was not confirmed until the causative agent was isolated on the rainbow trout (RT-2) cell line in 1963 (Jensen 1965). The virus was initially named Egtved virus, after the Danish village from which the isolate originated (Jensen 1965), however it was later renamed viral hemorrhagic septicemia virus (VHSV) by the World Animal Health Organization (OIE) (Altara 1963, Tordo et al. 2004). Koch's postulates were fulfilled following a series of

infection studies, including those by Yasutake and Rasmussen (1968) and Ord et al. (1976). Through the 1970's, VHSV was believed to be limited to freshwater rainbow trout farms in Europe (Kim and Faisal 2011).

In 1988, VHSV was isolated from spawning salmon in the state of Washington, USA during routine health examinations (Brunson et al. 1989, Hopper 1989). This was the first report of VHSV outside of Europe (Meyers and Winton 1995). Subsequent isolations in the Pacific Northwest from salmon (Stewart et al. 1990, Eaton and Hulett 1991) and Pacific cod (Meyers et al. 1992) documented the widespread distribution of asymptomatic marine carriers in the region. These findings prompted increased testing of marine fish populations worldwide. From this testing, it is clear that VHSV is common in major marine fisheries, including Atlantic cod in the North Sea (Jorgensen and Olesen 1989, Smail 1995), haddock and turbot in Scotland (Ross et al. 1994, Smail 2000), numerous species in the Baltic Sea, (Schlotfeldt et al. 1991), and flounder in the Western Pacific waters of Japan and Korea (Takano et al. 2001, Kim et al. 2003). A survey by Hedrick et al. (2003) found VHSV to be widespread in small prey species, such as herring and sardines, from southern California to Alaska on the Pacific coast of the US. Additional isolates from VHSV-positive fish were reported off the Northwest Atlantic coast of New Brunswick, Canada (Gagne et al. 2007). The consensus until 2004 suggested VHSV was widespread, was of marine origin, from which it was introduced to freshwater European rainbow trout farms during the mid-1900's (Einer-Jensen et al. 2004).

In 2005, VHSV was reported in the Laurentian Great Lakes (Lake St. Clair) basin in a dead muskellunge (Elsayed et al. 2006). This was a landmark event in several regards: 1) presence of VHSV in a North American freshwater waterbody, 2) susceptibility of this particular host species, and 3) presence of a fish rhabdovirus in the Great Lakes basin (Kim and Faisal 2011). Further investigation identified a VHSV isolate archived in 2003 from the same host species and waterbody. Subsequent reports from mortality events and survey studies documented the rapid expansion of VHSV from this initial infection site into all of the Great Lakes (Lumsden et al. 2007, Groocock et al. 2007, Bain et al. 2010, Kim and Faisal 2011). In addition, VHSV has been reported from inland lakes of Michigan, New York, Ohio, and Wisconsin (Figure B.2) (Thompson et al. 2011). The virus has proven to be highly pathogenic in this new environment, naturally infecting 28 different fish species to date and causing significant mortality as it has moved through the Great Lakes basin (USDA-APHIS 2008, Kim and Faisal 2011, Thompson et al. 2011). Although confirmed positive for VHSV by surveys of apparently healthy fish, Lake Superior has not had any documented mass mortality events similar to those in all other Great Lakes (Bain et al. 2010, Thompson et al. 2011, Faisal et al. 2012).

Figure B.2. Distribution of viral hemorrhagic septicemia virus in the Great Lakes region as of April 2010. Map provided by the USDA.



Advancements in molecular technology have allowed researchers to accurately group various VHSV isolates and describe the evolution of the virus worldwide. There are four recognized VHSV genotypes, loosely associated with geographical distribution, including I-III from Europe and IV from North America (Snow et al. 2004, Einer-Jensen 2004, Thompson et al. 2011). Genotypes Ib and IVa have also been reported in marine waters of Japan (Einer-Jensen 2004, Pierce and Stepien 2012). Analysis of the entire G

gene sequence, found the diversity within groups I, II, and III to be 1-6%, 1%, and 1-4%, respectively, and between the European genotypes to be 9-13% (Einer-Jensen 2004). The divergence between the European isolates and the North American isolates is 13-16%, which correlates to a divergence date of approximately 500 years ago (Einer-Jensen 2004).

In North America, the VHSV genotype IV contains three subgroups: IVa-c. Subgroup IVa contains all of the marine isolates from the Pacific Northwest, while IVb contains all of the freshwater isolates from the Great Lakes. A third subgroup, IVc, has been proposed by Pierce and Stepien (2012) and contains the marine isolates from the North Atlantic. The Great Lakes strain, IVb, differs from IVa by 3.6-4.6% and from IVc by 2.1-2.7% (Thompson et al. 2011). The genetic diversity of VHSV-IVb within the Great Lakes is very low. A recent analysis of 108 independent isolates by Thompson et al. (2011) found 61 to be identical to the index isolate (MI03) and 47 differed by only 1 nucleotide. Only 2% of all available VHSV-IVb isolates varied by more than 0.15%, and none greater than 1.05%. These findings suggest VHSV-IVb is a recent introduction and likely originated from the North Atlantic coast of North America (Thompson et al. 2011, Pierce and Stepien 2012). Further analysis of additional isolates (as they become available) and greater genome coverage will help clarify phylogenetic studies.

B.3 Clinical signs and pathology

The VHSV has a predilection for endothelial cells, and commonly induces hemorrhagic lesions throughout the body (Smail 1999, Brudeseth et al. 2002). Grossly,

this can be observed in the eye, skin, muscle, and internal organs (Figure B.3). In addition, VHS can be characterized by pale gills, lethargy, and abnormal swimming behavior (Wolf 1988). These findings have been reproduced experimentally with numerous infection studies (i.e. Kim and Faisal 2010). However, these lesions and signs should be interpreted with caution as they are not consistent across host species (Brunson et al 1989) and may be induced by other pathogens (i.e. Blazer et al. 2006).

Figure B.3. Bluegill experimentally infected with viral hemorrhagic septicemia virus. Note the periocular and dermal hemorrhages consistent with clinical lesions of VHS.

The precise route of viral entry is not fully understood; however, initial replication has been identified in gill epithelial cells (Chilmoncczyk 1980, Neukirch 1984, Brudeseth et al. 2008) and the base of fins (Harmache et al. 2006). During the first one to four days following infection the virus moves via the blood stream targeting endothelial cells of the kidney and spleen (Yasutake and Rasmussen 1968, Wolf 1988). At these locations, the virus causes necrosis and degeneration of hematopoietic tissues

and macrophage proliferation within renal tissue (Wolf 1998, Brudeseth et al. 2002, Kim and Faisal 2011). By day four to seven, multifocal hepatic degeneration and vacuolization of the liver is observed (Yasutake and Rasmussen 1968, Kim and Faisal 2010). Interestingly, despite this clear effect on hepatic morphology, Evensen et al. (1994) and Brudeseth et al. (2002) have shown liver endothelial cells are not directly affected by VHSV and, thus, may lack the appropriate receptors for viral attachment. In skeletal muscle tissue, erythrocyte aggregations are frequently observed between muscle fibers without significant damage to the surrounding tissue (Kim and Faisal 2010).

The clinical manifestation of VHS is dependent on the form of the disease. Acute disease results in rapid destruction of endothelial cells and extravasation of the blood supply, resulting in organ failure, anemia, and high mortality (Kim and Faisal 2011). Chronically infected fish will often experience a prolonged disease that can lead to neurologic lesions characterized by erratic swimming behavior (Yasutake 1975). In addition, these fish can become long term shedders of the virus into the surrounding environment (Neukirch 1985). Morbidity and mortality from VHSV vary greatly among species and depend, in part, on host immune response and environmental conditions; however, VHSV is generally considered to be a highly lethal disease with 100% mortality for fry and 25-90% for adult fish (Meyers and Winton 1995, Skall et al. 2005, OIE 2009).

B.4 Transmission

The natural transmission of VHSV has largely been informed by studies on European freshwater fish populations (OIE 2009) that have then been recently applied to

the Great Lakes region (VHSV Expert Panel 2010). The virus is primarily shed into the water from infected fish via excretory and reproductive fluids (Chilmonczyk 1980), providing both horizontal and vertical routes for transmission. Thus, cohabitation and natural dispersal in connected waters are the most common routes of transmission (VHSV Expert Panel 2010). However, the movements of contaminated water or fomites via recreation, industry, and natural resource activities are likely to have contributed to widespread dispersal of the virus (Bain et al. 2010, Herve-Claude et al. 2008, VHSV Expert Panel 2010, Phelps et al. in press). Other possible, but less likely, vectors include piscivorous birds (Peters and Neukirch 1986), turtles (Goodwin and Merry 2011a), and amphipods (Faisal and Winters 2011).

Water temperature is a significant factor for survivability, and thus, transmissibility of VHSV in the environment (Hawley and Garver 2008, OIE 2009, Goodwin and Merry 2011b, Goodwin et al. 2012). Ideal water temperatures for VHS occur from 9-12°C (Smail 1999, Hedrick et al. 2003, Kim and Faisal 2010, Eckerlin et al. 2011). Consequently, increased transmission and outbreaks are more frequently observed in the spring when optimal water temperatures coincide with physiologic stress associated with spawning (Wolf 1988, OIE 2009). A study by Hawley and Garver (2008) investigating the viability of VHSV in fresh and saltwater emphasized the influence water temperature has on viral persistence in the environment. In filtered freshwater at 4°C, VHSV-IVb remained infective for more than 1 year, but infectivity was eliminated after 12 d and 1d at 20°C and 30°C, respectively. Water quality was also a significant contributor to survivability, with infective virus found only between 27 and 40 days at

4°C in unfiltered freshwater. Interestingly, VHSV-IVb remained viable as long as marine strains in saltwater. The poor survivability observed by Hawley and Garver (2009) at warmer temperatures is supported by in vivo and in vitro studies. In cell culture, temperatures above 15°C are inhibitory to virus growth, and, in the host, disease rarely occurs above that temperature (Clark and Soriano 1974, de Kinkelin et al. 1980, Wolf 1988, Winton et al. 2007, Goodwin and Merry 2011b). Low water temperatures, 1 - 5°C, will typically produce a persistent and chronic disease course, with low daily mortality resulting in carrier fish (OIE 2009, Kim and Faisal 2011).

B.5 Detection and identification methods

Many of the lesions observed for VHSV are not pathognomonic. Consequently, the development of diagnostic methods for the detection and identification of VHSV in fish has been an area of significant effort. Methods that have undergone stringent validation processes and are accepted by international standards are included in the OIE's Manual of Diagnostic Tests for Aquatic Animals (OIE Manual; 2009) and the American Fisheries Society – Fish Health Section's Blue Book (Blue Book; 2010). These methods, included in the following review, are widely used by aquatic diagnostic laboratories for the primary detection and confirmation of VHSV.

B.5.1 Cell culture. Viral hemorrhagic septicemia virus isolation in cell culture is the 'gold standard' diagnostic method for regulatory testing and is widely used for surveillance programs (OIE 2009, USFWS and AFS-FHS 2010). Several suitable cell

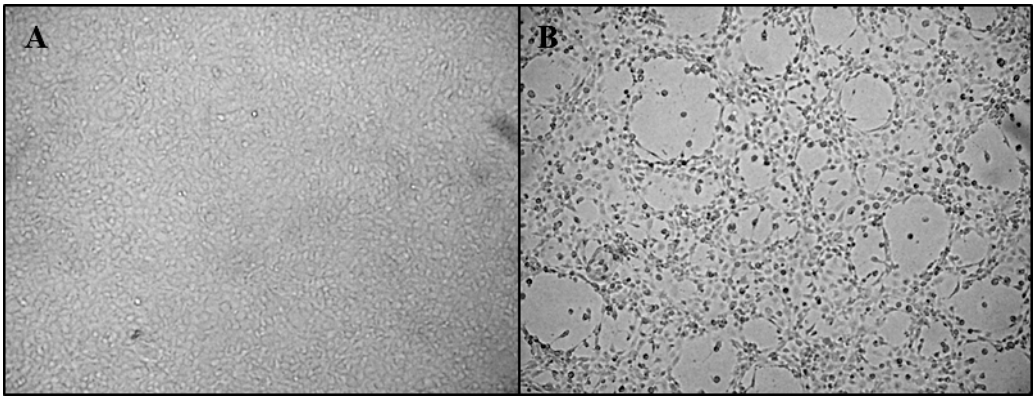
lines are available for culture, including: rainbow trout gonad (RTG-2), bluegill fry (BF-2), chinook salmon embryo (CHSE-214), *epithelioma papulosum cyrpini* (EPC), and fathead minnow (FHM) cells (OIE 2009, USFWS and AFS-FHS 2010). Cell line sensitivity to various VHSV strains does vary. RTG-2 and BF-2 were most effective for European isolates, but showed limited sensitivity for the North American strains (Lorenzen et al. 1999, Winton et al. 2010). EPC and FHM cell lines have been shown to consistently perform well, with high sensitivities for all virus strains (Winton et al. 2010). It was recently reported the EPC and FHM cell lines commonly used in the United States are, in fact, both FHM (Winton et al. 2010). The precise contamination event has not been determined, however a broad survey of EPC cells suggests this occurred near its origin and has been widely distributed (Winton et al. 2010).

The type of sample suitable for cell culture is dependent on the size of the fish. Although it would be ideal to target tissues with high viral replication (i.e. kidney, spleen, heart) the small quantity of tissue available in small fish makes this infeasible. The OIE Manual and Blue Book provide the following sampling recommendations: from large fish (> 6 cm) collect kidney, spleen, heart, encephalon; from fish 3 – 6 cm collect entire viscera; from small fish (< 3 cm) collect whole body; and from spawning fish collect ovarian fluid (OIE 2009, USFWS and AFS-FHS 2010).

Processing tissue samples for cell culture typically follows the methods established by the OIE Manual and the Blue Book (OIE 2009, USFWS and AFS-FHS 2010). Cell cultures are inoculated with clarified tissue suspension and incubated at 15°C for 7-14 days. Unless cytopathic effects (CPE) are observed, the sample is blind

passaged for an additional 7-14 days. The sample is determined negative if no CPE develops during the second passage. Cytopathic effects consistent with VHSV include cell rounding and aggregation, degeneration, and vacuolization (Figure B.4). Similar CPE is produced by other rhabdoviruses and should therefore be pursued by secondary tests to confirm the presence of VHSV.

Figure B4. A) Normal epithelioma papulosum cyrpini (EPC) cell line and, B) EPC cell line with cytopathic effects characteristic of rhabdoviruses, including viral hemorrhagic septicemia virus.

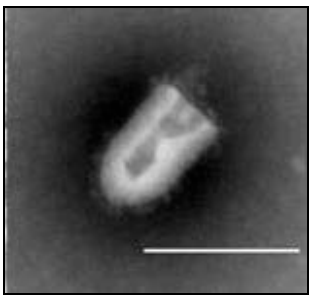


B.5.2 Electron microscopy. Visualization of VHS viral particles by negative contrast electron microscopy can be a useful diagnostic screening tool (Zwillenburg et al. 1965, Granzow 1997, Elsayed et al. 2006). However, VHSV cannot be clearly distinguished from other rhabdoviruses in fish by this method (Granzow et al. 1999). As with any diagnostic assay, the quality of the sample is imperative. A purified cell culture supernatant that exhibited CPE is ideal, with a high likelihood of confirmation (Elsayed

et al. 2006). Negative contrast electron microscopy can also be performed directly on tissue or feces; however, the virus must be present in high levels to be detected.

Processing samples for electron microscopy is a delicate procedure, as fine ultrastructural characteristics can be easily altered. If not immediately processed by an electron microscopy laboratory, samples should be preserved in 10% neutral buffered formalin or, preferably, 2.5 – 3.0% glutaraldehyde. A variety of laboratory processing methods are used to prepare the sample for visualization, however the ultimate goal is to thoroughly fix the sample, coat with a stain, and subject it to an electron beam. The resulting image is then observed through a high powered microscope. Electron dense regions appear lighter in color, as seen in Figure B.5.

Figure B5. Rhabdovirus particle observed by negative contrast electron microscopy, approximately 180 nm x 70 nm. Scale bar = 200 nm.



B.5.3 Antibody-based assays. Several antibody-based detection methods are recognized by the OIE Manual and Blue Book, such as indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) (Mourton et al. 1992, OIE 2009, USWFS and AFS-FHS 2010). Imprints or sections from kidney or liver are ideal

tissue samples during acute infection and can provide a more rapid result compared to virus isolation in cell culture (Evensen et al. 1994). However, these methods are limited by poor sensitivity and lack of specificity of the antibodies and results should be interpreted with caution (Evensen et al. 2004, OIE 2009).

For surveillance testing, direct identification of VHSV can be problematic if virus levels are low (common in carrier, subclinical, or recovered fish) and generally requires lethal tissue collection. In contrast to the comparably short-lived virus in infected tissues, neutralizing antibodies can be measured in blood longer following infection (LaPatra 1996, Millard and Faisal 2012a), but do not necessarily diagnose active infection. This was demonstrated by a recent serological survey of Lake St. Clair, a VHSV-IVb endemic waterbody. Neutralizing antibodies were detected in serum samples from several fish species, without positive isolation (Millard and Faisal 2012b).

B.5.4 Molecular assays. The use of rapid and ultrasensitive methods for the detection of VHSV is valuable to effectively manage the disease in farm raised and wild fish populations. Consequently, the development of highly sensitive molecular methods, including reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (rRT-PCR) is becoming more important (Walker 2002, Purcell et al. 2011, Bland et al. 2012). Both RT-PCR and rRT-PCR methods exist for the detection of VHSV in fish (Winton and Einer-Jensen 2002, Einer-Jensen et al. 2005, Hope et al. 2010, Garver et al. 2011, Jonstrup et al. 2012, Phelps et al. 2012). These assays have been validated to varying standards and have been designed for specific purposes. For example, RT-PCR

is more commonly used for confirmatory testing following virus isolation (OIE 2009, USFWS and AFS-FHS 2010). Three of the published rRT-PCR assays were designed to detect all known strains of VHSV (Garver et al. 2011, Jonstrup et al. 2012, Phelps et al. 2012), while another assay was optimized to detect VHS-IVb (Hope et al. 2010). All of these assays have demonstrated their value as a diagnostic surveillance tool by increasing sensitivity and specificity, and significantly reducing turnaround time compared to virus isolation (Knuesel et al 2007, Bain et al. 2010, Hope et al. 2010, Garver et al. 2011, Jonstrup et al. 2012, Phelps et al. 2012). However, molecular methods are not recognized by the OIE Manual or Blue Book for primary diagnostic testing and thus, widespread acceptance among regulatory agencies remains limited (OIE 2009, USFWS and AFS-FHS 2010, Purcell et al. 2011). In Minnesota, the use of rRT-PCR is allowed for the movement of wild caught baitfish; however, all fish must also be inspected by virus isolation (Minnesota Department of Natural Resources, personal communication).

Until the regulatory framework is established, the voluntary use of rRT-PCR for the detection of VHSV can significantly improve management of VHSV. For example, prior to 2010, the majority of Minnesota waterbodies used for aquaculture or recreational purposes had never been tested for VHSV. As a result, the VHS status for much of the state was unknown, despite routine movements of fish between waterbodies for bait and stock enhancement. The survey performed as part of this dissertation using rRT-PCR (Phelps et al. 2012) has better informed epidemiologists and managers on the extent of the virus presence in Minnesota.

B.6 Prevention and control

B.6.1 Therapeutics. Widespread vaccination programs are not currently used for the prevention of VHS (Sommerset 2005). The challenges of vaccine development for fish were highlighted more than thirty years ago by de Kinkelin et al. (1981) and remain true today (Sommerset et al. 2005). de Kinkelin et al. (1981) indicated a vaccine must meet several criteria, including 1) a single dose administration, 2) easy delivery, 3) reasonable cost, 4) delivery of universal protection, 5) no risk of mutation, 6) remaining stable in storage, and 7) not interfering with sero-surveillance programs. Several factors compound these challenges in aquatic systems, including the aqueous environment, the comparably lower economic value of fish, and limited understanding of the host immune system, compared to terrestrial animals. Significant efforts have been made to meet these criteria for a VHSV vaccine in recent years (Kim and Faisal 2011). A number of vaccines, including inactivated, live attenuated, and recombinant versions, have been developed and evaluated for effectiveness against VHSV infection in rainbow trout; however, efficacy and delivery have remained problematic (Lorenzen et al. 1993, Lecocq et al. 1994, de Kinkelin 1981, Kim and Faisal 2011). Perhaps most promising are advancements in DNA vaccines (Robinson and Pertmer 2000). Einer-Jensen et al. (2009) developed a dual DNA vaccine targeting the glycoprotein gene of VHSV and IPNV, which was shown to provide long-term protection. This protection was attributed to stimulation of the adaptive immune system (Einer-Jensen et al. 2009, Hart et al. 2012). Thus, vaccine science for fish is advancing, but efforts are still at a rudimentary level compared to vaccines in terrestrial species. As understanding of the immune system,

host-pathogen interaction, physiology, and environmental influences in fish improve, so too will the ability to develop and administer effective therapeutics.

Given the rudimentary status of vaccination development, the best existing approach to prevent the spread and transmission of VHSV is to eliminate the transfer of the virus via equipment, eggs, fish, and contaminated water. Numerous disinfectants are effective to inactivate VHSV, including chlorine, hypochlorite, formalin, and sodium hydroxide (Wolf 1988, Smail 1999). Ultraviolet (200-280 nm wavelength) irradiation is also frequently used to treat incoming surface water for aquaculture facilities (Oye and Rimstad 2001). Since the 1970's, iodophor treatment of salmonid eggs has been a standard hatchery practice to prevent vertical transmission of pathogens, like VHSV, on the surface of the egg (Tuttle-Lau 2009). These methods have been more recently evaluated for use on walleye and northern pike eggs in the Great Lakes region. A recent study by Tuttle-Lau et al. (2009) demonstrated 100 ppm iodophor treatments either at 5 min after fertilization for 30 min or at 90 min after fertilization for 10 min successfully eliminated VHSV-IVb from the surface of walleye and northern pike eggs without inhibiting hatching success. These methods have become standard practice for aquaculture production in the Great Lakes region. However, the aforementioned approaches cannot be effectively administered to wild populations which are at risk of unavoidable natural dispersal. This concern highlights the importance of preventing the widespread dissemination of VHSV by aquaculture and recreational activities.

B.6.2 Regulations. Prior to 2007, the non-salmonid aquaculture industries in the United States were largely unregulated. As with other significant emerging disease threats, the regulatory reaction to VHSV-IVb in the Great Lakes was swift and strict. In 2007, the USDA-APHIS created a Federal Order limiting the movement of fish within and out of the Great Lakes region. The Order was later amended in 2008, in part due to industry backlash, as well as improved knowledge of the disease (USDA 2008b). Briefly, the Federal Order 1) defines positive or at-risk states to include all states within the Great Lakes watershed, 2) defines VHSV-susceptible species as those found positive during a natural outbreak by cell culture, and 3) requires annual inspection of live VHSV-susceptible species transported across state boundaries. Much has been learned regarding the distribution, host susceptibility, and environmental requirements specific to VHSV-IVb since 2008 (Kim and Faisal 2011); however, further modifications to the Federal Order have been postponed indefinitely. In addition to the federal regulations, states have developed state-specific regulations based their unique hazards and management goals for the disease (Focus on Fish Health 2012). This dizzying array of regulations is beyond the focus of this dissertation; however it offers an example of the current challenges to manage VHSV in the Great Lakes region.

As our knowledge of VHSV continues to grow, so too must our ability to manage it. Several strategies have been proposed to combine scientific findings and policy. Amos et al. (1998) performed a retrospective analysis of VHSV-IVa management in the Pacific Northwest, finding it imperative to implement an “adaptive management strategy”. Routinely evaluating management decisions based on current understanding,

not political or industry pressures, will improve the success of control programs. In addition, the use of a "risk-based management strategy" has been proposed by the VHSV Expert Panel and Working Group (2010) and Gunderson et al. (2010). They argue that VHSV prevention based on political boundaries is not an effective management strategy compared to focusing on watersheds or areas of risk.

B.7 Conclusion

Despite the importance of viral hemorrhagic septicemia in the Great Lakes region, much of what we know about the disease has been derived from marine populations and farm raised rainbow trout of Europe. While lessens have been learned, VHSV-IVb poses unique challenges that must be addressed, including the most fundamental diagnostic and management strategies. Through the following five studies, this dissertation has advanced the understanding of VHSV-IVb, improved the ability to respond, and informed management recommendations.

SECTION C: IMPROVED DIAGNOSIS

CHAPTER 1

THE USE OF A ONE-STEP rRT-PCR FOR THE SURVEILLANCE OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS IN MINNESOTA

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Viral hemorrhagic septicemia virus (VHSV) is a highly contagious and pathogenic virus of fish. The virus infects more than 70 fish species worldwide, in both fresh and salt water. A new viral strain (VHSV-IVb) has proven both virulent and persistent, spreading throughout the Great Lakes of North America and to inland water bodies in the region. To better understand the geographic distribution of the virus, we used a modified real time reverse transcription-polymerase chain reaction (rRT-PCR) assay for high-throughput testing of fish for VHSV. The assay was shown to be twice as sensitive as the gold standard, virus isolation, and did not cross react with other viruses found in fish. In addition, the diagnostic turnaround time was reduced from 28-30 days for virus isolation to 2-4 days for rRT-PCR. To demonstrate the usefulness of the rRT-PCR assay, 115 high priority water bodies in Minnesota were tested by both methods from April 2010 – June 2011. All survey sites tested negative for VHSV by both methods. The survey results have informed fisheries managers on the absence of VHSV in Minnesota and have better prepared them for the eventual arrival of the disease. In addition, the results demonstrate the value of this rRT-PCR as a surveillance tool to rapidly identify an outbreak so that it can be controlled in a timely manner.

1.1 Introduction

Viral hemorrhagic septicemia virus (VHSV) is a highly contagious and pathogenic virus of fish. The virus has a broad host range including more than 70 fish species and is capable of causing significant biological and economic losses (Kim and Faisal 2011, OIE 2009). The presence of this virus was first suspected as early as 1938, but was not confirmed until 1963 when VHSV was isolated from freshwater rainbow trout *Onchorhynchus mykiss* farms in Denmark (Shaeperclaus 1938, Jenson 1963). Currently, there are four genotypes of VHSV (VHSV-I to VHSV-IV). Genetic analysis of viral isolates through the 1990s from Europe (VHSV-I, II, III), East Asia (VHSV-I, III, IV), and North America (VHSV-IV) concluded that VHSV is of marine origin but has also mutated to cause disease in freshwater fish hosts (Einer-Jensen et al. 2004, Kim and Fiasl 2011, Pierce and Stepien 2012). The routes of transmission between regions and environments are not fully known, but the transfer of infected water, baitfish, and fish for stock enhancement are likely candidates (Bain et al. 2010, Herve-Claude et al. 2008, VHS Expert Panel 2010).

In 2003, VHSV was isolated in the Laurentian Great Lakes (Lake St. Clair) basin from a muskellunge *Esox masquinongy*. This was a first event in several regards: 1)

presence of VHSV in freshwater of North America, 2) susceptibility of this particular host species, and 3) presence of fish rhabdovirus in the Great Lakes basin (Kim and Faisal 2011). Unfortunately, this isolate was not confirmed to be VHSV until 2005 during a second muskellunge mortality event (Elsayed et al. 2006). Since then the virus has proven to be highly pathogenic in this new environment, naturally infecting 28 different fish species and causing significant losses throughout much of the Great Lakes basin (Kim and Faisal 2011, Thompson et al. 2011, USDA-APHIS 2010). Although confirmed positive for VHSV by surveys of apparently healthy fish, Lake Superior has not experienced mass mortality events similar to those in the Eastern Great Lakes (Bain et al. 2010, Thompson et al. 2011).

Within the North American VHSV genetic group IV, the marine and freshwater isolates are grouped into VHSV-IVa and VHSV–IVb, respectively (Meyers and Winton 1995). The genetic divergence within VHSV-IVb is very low (maximum of 1.05 % in 669 nt of the G-gene), which is consistent with a recent introduction of the virus in a naive population (Thompson et al. 2011, Pierce and Stepien 2012). Although the differences were minor, Thompson et al. (2011) did identify multiple unique isolates in single outbreak locations, including inland lakes. This has supported the covert nature of the virus and its ability to repeatedly move or be introduced undetected. Therefore, continued virus surveillance is critically important to reduce further spread of VHSV within, and out of, the Great Lakes basin.

Several methods are currently utilized for regulatory, clinical, and surveillance testing of VHSV. Virus isolation is the gold standard diagnostic assay for all fish viruses

as recommended in the Blue Book (USFWS-AFS FHS 2010) and the Manual of Diagnostic Tests for Aquatic Animals (OIE 2009). As such, virus isolation is widely used and accepted for regulatory testing of fish to certify virus-free status (Purcell et al. 2011). However, for surveillance or other voluntary testing without movement or legal implications, the selection of diagnostic tests can be much more flexible. While an assay for surveillance purposes must be validated and proven robust to high standards, it should be best fit for the purpose. One method that has gained popularity in recent years for surveillance testing is real time polymerase chain reaction (rt-PCR) (Makay et al. 2002, Walker 2002, Espy et al. 2006). It has often been shown that the rt-PCR improves sensitivity, speed, and accuracy, while lowering cost, compared to traditional methods of virus isolation (Leland and Ginocchio 2007, Hope et al. 2010). These advantages allow managers to have a better understanding of virus distribution and to more rapidly respond to an outbreak. Limitations, such as poor validation and risk of cross contamination in molecular assays have been significantly reduced with increases in laboratory experience and technological advancements (OIE 2009, Purcell 2011).

Real time PCR has been widely used and federally recognized as a primary detection and surveillance assay for a variety of terrestrial animal diseases, including: avian influenza, exotic Newcastle disease, classical swine fever, swine influenza, foot and mouth disease, and many others (OIE 2009, USDA-APHIS 2010, FDA 2011). While the same cannot be said for rt-PCR for aquatic pathogens, there has been widespread development/validation for their use in research and clinical cases (Purcell et al. 2011). Both conventional reverse transcription PCR (RT-PCR) and real time RT-PCR (rRT-

PCR) methods exist for the detection of VHSV in fish (Winton and Einer-Jensen 2002, Hope et al. 2010, Garver et al. 2011, Jonstrup et al. 2012). These assays have been validated to varying standards and have been designed for specific purposes. For example, conventional RT-PCR is more commonly used for confirmatory testing following virus isolation (OIE 2009, USFWS-AFS FHS 2010). Two of the previously published rRT-PCR assays were designed to detect all known strains of VHSV (Garver et al. 2011, Jonstrup et al. 2012), while another assay was designed to detect only the VHS-IVb strain in the Great Lakes (Hope et al. 2010). All of these assays have demonstrated their value as a surveillance tool by increasing sensitivity and significantly reducing turnaround time compared to virus isolation (Knuesel et al 2007, Bain et al. 2010, Hope et al. 2010, Garver et al. 2011, Jonstrup et al. 2012).

Until the regulatory framework is established, the voluntary use of rRT-PCR for the detection of VHSV can significantly improve management of this virus. For example, prior to 2010, the majority of Minnesota waterbodies used for aquaculture or recreational purposes were never tested for VHSV. As a result, the VHS status for much of the state was unknown, despite routine movements of fish between waterbodies for bait and stock enhancement. In the present study, a one-step version of the Garver et al. (2011) assay was used for high-throughput testing of fish for VHSV surveillance. This initial survey has informed epidemiologists and managers on the extent of the virus presence in Minnesota, which has better prepared them for the eventual arrival of VHSV. In addition, the survey has satisfied the necessary regulatory testing requirements for

aquaculture producers to move fish within the state (Minnesota statutes 2011, Section 17.4991, subdivision 3).

1.2 Methods

1.2.1 Source of samples. A survey of Minnesota fish populations was performed for the presence of VHSV from April 2010 through June 2011. Registered license holders for aquaculture facilities (n = 145) and baitfish harvesters (n = 289) were contacted by mail to participate in the surveillance program. The program offered a free "regulatory inspection", which consisted of collecting fish and testing them simultaneously by virus isolation (the gold standard test) and modified rRT-PCR. Fish species included in the survey were limited to those on the USDA-APHIS VHSV susceptible species list (2008) at the time of the survey. Sample locations were selected based on 1) waterbody use by the aquaculture industry, 2) presence of VHSV susceptible species, 3) distribution throughout Minnesota, and 4) high risk/high priority as identified by the Minnesota Department of Natural Resources (MNDNR).

On-site fish collection was overseen by an accredited veterinarian or MNDNR field biologist with specialized fish health training, or an American Fisheries Society Fish Health Inspector. A minimum of 60 fish per site were collected, targeting species on the aquaculture permit, such as the spottail shiner *Notropis hudsonius* and walleye *Sander vitreus*. If the target species was unattainable due to seasonal availability, 150 fish of other VHSV susceptible species were collected. For large populations (>1,000 fish), sampling of 60 fish provided 95% confidence in detecting VHSV, assuming the virus

was present in 5% of the population. The sample of 150 susceptible fish maintained 95% confidence, but accounted for a potentially lower prevalence of 2%. Fish collections primarily occurred during the spring and fall when water temperatures were suitable for VHSV.

Freshly dead fish were submitted on ice to the Minnesota Veterinary Diagnostic Laboratory (MVDL) within 24 hours of collection. Immediately upon arrival, each fish was visually inspected for clinical signs of disease and a necropsy was performed to obtain the appropriate samples e.g., kidney/spleen from > 6.0 cm fish, entire viscera from 4.0 – 6.0 cm fish, and the entire fish when it was < 4.0 cm in length. Tissues from five fish were pooled together and stored at 4°C for no more than 24 hours. The pools were tested for VHSV by both virus isolation and rRT-PCR. Results were made immediately available online for the owner and the regulatory agency for review.

1.2.2 Sample preparation. Due to regulatory implications of the survey results, all samples from this study were tested by both virus isolation and rRT- PCR. Tissue processing was identical for both methods and followed the protocols recommended by USFWS and AFS-FHS Blue Book (2010). Briefly, a 10% suspension of the tissue was prepared in Hanks' Balanced Salt Solution (HBSS). The suspension was processed in a stomacher for 30 sec followed by centrifugation at 4°C for 15 min at 2,900 xg. An equal amount of antibiotic incubation medium was added to the supernatant. Following mixing, the suspension was incubated for 2 hrs at 15°C and finally re-centrifuged for 15

min at 2,900 xg. The resulting supernatant was immediately used for virus isolation and rRT-PCR.

1.2.3 Virus isolation. Virus isolation was performed according to the USFWS and AFS-FHS Blue Book (2010). Monolayers of epithelioma papulosum cyprini (EPC) cells (Fijan et al. 1983) were prepared in 48-well microtiter plates. When monolayers were 80% confluent, the cell culture media was decanted and the sample was inoculated (100 µl/well). The inoculated cells were incubated at 15°C for 60 min to allow for viral adsorption. Maintenance medium was then added and the plates incubated at 15°C for 14 days. The plates were examined twice weekly for the appearance of cytopathic effects (CPE). Unless CPE was observed, all samples were blind passaged at day 14 and determined negative if no CPE appeared after 14 days of incubation. A VHSV-IVb isolate (Great Lakes reference strain: MI03) was used as a positive control to monitor the sensitivity of the cell line to the virus. The CPE from the positive control was occasionally confirmed by rRT-PCR, sequencing, and negative contrast electron microscopy. Cell culture supernatant was stored at 4°C for no more than 24 hours prior to extraction.

1.2.4 Total nucleic acid extraction. MagMAX Express 96 Viral RNA Isolate kit (AM1836; Life Technologies, Grand Island, NY) was used for total nucleic acid extraction from sample suspension and from infected cell culture supernatants. The extraction was performed according to a custom protocol developed by the MVDL in

association with Life Technologies (Grand Island, NY). The primary difference from the standard protocol is a 100 µl starting volume and 75 µl final elution volumes, compared to 50 µl for each in the standard protocol. This method was originally optimized for high-throughput extraction of a swine virus, the porcine reproductive and respiratory syndrome virus (PRRSV), and has been found to be effective for a wide variety of other animal pathogens (data not shown). Along with unknown samples, a VHSV-IVb positive isolate grown in EPC cells was extracted for each run to serve as a positive extraction control. The extraction procedure was also performed on a 100µl 1X PBS to serve as a negative extraction control. The resulting RNA was stored on ice for immediate testing or frozen at -80° C for later testing.

1.2.5 Real time reverse transcription polymerase chain reaction. Real time RT-PCR for VHSV was performed on an ABI 7500 Sequence Detection System (Life Technologies, Grand Island, NY). The master mix was prepared in a 20 µl volume, with 12.5 µl 2X Path-ID Multiplex RT-PCR Buffer (Life Technologies), 1.25 µl 10X Path-ID Multiplex enzyme mix (Life Technologies), 1.0 µl of 5 µM FAM labeled – MGB Probe (5'-Fam-TAC GCC ATC ATG ATG AGT- 3'), 0.375 µl of each 40 µM forward (5'-ATG AGG CAG GTG TCG GAG G-3') and reverse (5'-TGT AGT AGG ACT CTC CCA GCA TCC-3') primers, and 4.5 µl of nuclease-free water. The primer/probe set was designed to detect the N-gene of all known strains of VHSV (Garver et al. 2011). Five µl of extracted RNA (unknown samples), nuclease free water (negative control), VHSV known-positive RNA (positive PCR control), VHSV known-positive RNA

(positive extraction control), or negative extraction control were added to each well. Each sample was run in duplicate. The thermal cycling protocol consisted of 10 min at 45°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C.

1.2.6 Analytical specificity and sensitivity. The specificity of the modified assay was confirmed with spring viremia of carp virus, infectious salmon anemia virus, channel catfish virus, koi herpes virus, golden shiner virus, two fathead minnow picornavirus, and an unknown paramyxo-like virus of white suckers. For sensitivity determination, serial dilutions of VHSV positive cell culture supernatant were tested in duplicate by virus isolation and rRT-PCR on four separate days. The lowest dilution that yielded a positive result was determined to be the limit of detection. The threshold cycle (Ct) for the rRT-PCR was considered as the cycle in which the amplification curve crossed the automatic threshold (Life Technologies, Grand Island, NY) during the exponential growth phase of the curve. The rRT-PCR reaction had a cut off at 40 cycles but all samples with Ct values 37 – 40 were automatically re-tested. If they remained below 40 cycles, they were called positive.

1.3 Results

1.3.1 VHSV Survey. Thirty aquaculture producers and wild baitfish harvesters participated in the VHSV survey out of 434 contacted. The industry impact was likely much higher given that once a waterbody was certified VHSV-free, anyone with the appropriate license could harvest bait. From April 2010 to June 2011, 115 water bodies

were tested for VHSV as part of this survey (Figure 1.1). The sampling locations with high aquaculture and wild baitfish harvest were well distributed across Minnesota. A total of 7,098 individual fish were collected, comprising 1,420 pooled samples. Approximately 56% (n = 65) of the surveyed sites consisted of sport fish, while 44% (n = 50) consisted of baitfish. Specific species included walleye *Sander vitreus* (n = 3,375), spottail shiner *Notropis hudsonius* (n = 3,000), black crappie *Pomoxis nigromaculatus* (n = 285), muskellunge (n = 265), northern pike *Esox lucius* (n = 80), bluegill *Lepomis macrochirus* (n = 60), yellow perch *Perca flavescens* (n = 18), and smallmouth bass *Micropterus dolomieu* (n = 15). All sample pools were found negative for VHSV by both virus isolation and rRT-PCR. Throughout the survey, the virus isolation and rRT-PCR assays were 100% accurate in identifying the positive and negative controls. The turnaround time for virus isolation and rRT-PCR was 28-30 days and 2-4 days, respectively.

1.3.2 VHSV rRT-PCR. Analytical specificity – The rRT-PCR was specific to VHSV and did not cross react with other viruses tested. Although the VHS-IVb isolate was the only VHSV tested in this validation, sequence alignment and previous data show the primers used can detect all known VHSV strains (Garver et al. 2011).

Analytical sensitivity – The analytical sensitivity of this assay was comparable to other rRT-PCR assays for VHSV. The standard curve was linear over six logs of virus dilution, with a starting concentration of 4.6×10^6 $TCID_{50}$ (Figure 1.2). The correlation coefficient and PCR efficiency were 0.9942 and 104.5%, respectively. The minimum

detection limit of the rRT-PCR assay was determined to be a 1.44 $TCID_{50}$ with a corresponding Ct of 37.2. Compared to this rRT-PCR assay, virus isolation was half as sensitive, with a detection limit of 2.88 $TCID_{50}$ and corresponding Ct of 34.8.

1.4 Discussion

A newly modified one-step rRT-PCR for the detection of VHSV was used in this study. The assay was shown to be twice as sensitive as the gold standard, virus isolation. Since VHSV can persist in wild fish populations at low levels without producing clinical signs of disease, this improved sensitivity is critically important to prevent the spread of VHSV. In addition, rRT-PCR can significantly decrease the turnaround time and lower labor and laboratory costs. The analytical data generated from this study show this assay to be an excellent candidate for further evaluation and fit for the purpose of surveillance testing as demonstrated in a two-year survey of Minnesota waters for VHSV. With nearly 1,500 sample pools tested, no false-positive results were obtained while both tests accurately identified the negative and positive controls. The results from this survey have certified 115 locations to be VHSV negative, allowing in-state fish movement (for one year post inspection).

Aquaculture industry support for this study was very strong in Minnesota, due to a desire to move fish within days of sample submission, rather than having to wait a month. This is essential for any industry that survives on quick turn-around and accurate test results. The survey data have informed management agencies and researchers of the distribution of VHSV in Minnesota. Applications for these data are wide-reaching and

will improve selection criteria for future testing, support risk-assessment studies, and ease public concern for this emerging fish disease.

Despite the aforementioned advantages, there are certain limitations of rRT-PCR. One significant limitation is the appropriate interpretation of results. If rRT-PCR is used for surveillance testing, positive rRT-PCR results should remain suspect until confirmed by virus isolation. If virus isolation does not support the suspect-positive result, it should be considered "a population of interest, in need of further testing" or "negative" as it would have been without the use of rRT-PCR. A suspect-positive by rRT-PCR should not result in immediate depopulation or other regulatory action. In the event of conflicting results, other factors should be considered to warrant additional action, such as clinical signs of disease, previous testing history, disease risk factors, or the number of suspect-positive results. Additional action may include, re-testing the original material, re-sampling the population, sequencing the rRT-PCR product, and other confirmatory tests.

One other limitation is proper validation and quality control in the laboratory. Validation standards are outlined by the OIE (2009) and Purcell et al. (2011). While the degree to which an assay is validated depends on its proposed use, the assays used for widespread surveillance testing should be thoroughly scrutinized. The assay used in this study, for example, should undergo an inter-laboratory validation to evaluate various platforms, technicians, and diagnostic sensitivity/specificity. Furthermore, proper controls should be used throughout the rRT-PCR assay, including positive and negative controls for RNA extraction and rRT-PCR. These controls were used in this study and

increased confidence in the interpretation of results. Endogenous controls were not used in this study, but are recommended to confirm the quality and quantity of nucleic acid (Bland et al. 2012). Other concerns such as laboratory conditions, experience, and capacity are rapidly improving in the United States as technology and demand increases.

Given the threat of aquatic animal pathogens, such as VHSV, new and improved diagnostic tests must be developed, validated, and recognized by agencies for use in surveillance testing. Rapid detection and quick turnaround time are paramount to identify and effectively control an outbreak. This need has been well demonstrated with the emergence of VHSV-IVb in the Great Lakes. As technology continues to improve and appropriate evaluations are performed, rRT-PCR will become the assay of choice for the detection of VHS and other emerging diseases of aquatic animals.

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Figure 1.1. Map of 115 VHSV survey locations in Minnesota collected from April 2010 – June 2011. Circles indicate sportfish (n = 65) and squares indicate baitfish (n = 50). All locations tested negative for VHSV by virus isolation and rRT-PCR. Image generated with Google Earth.

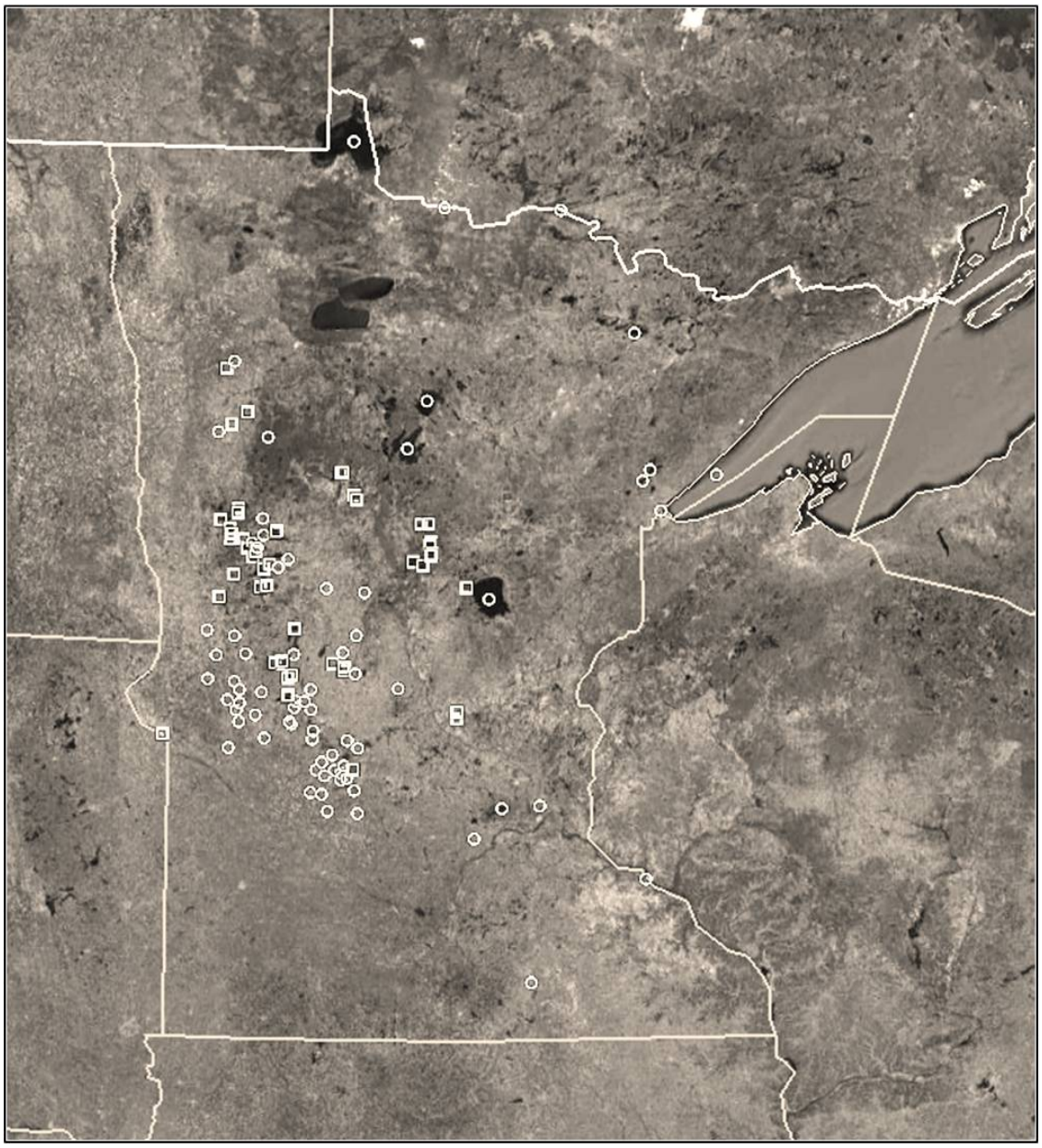
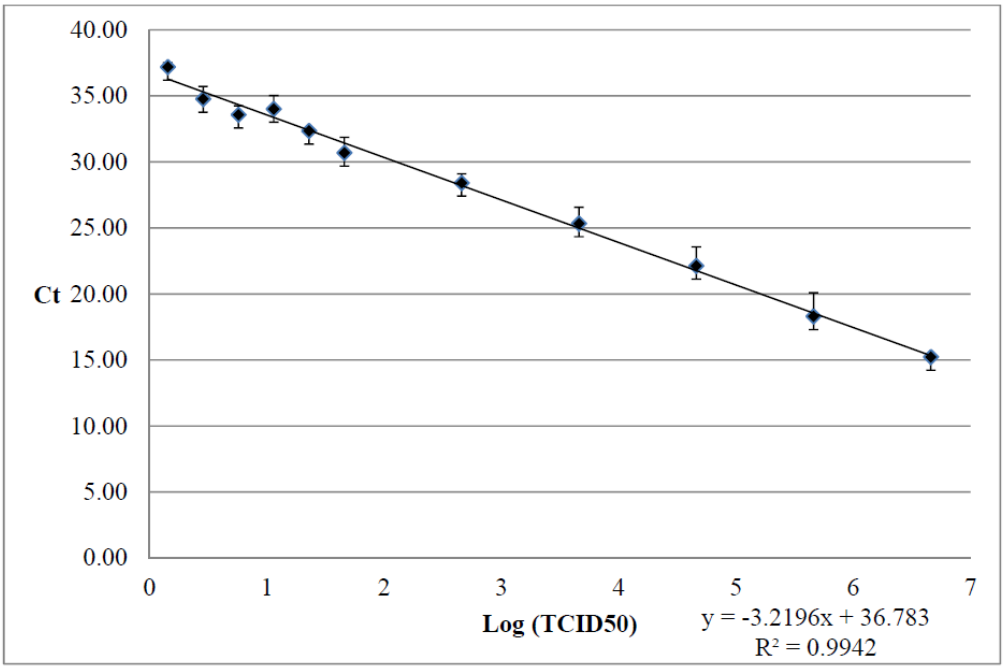


Figure 1.2. rRT-PCR standard curve with dilutions of VHSV, with initial concentration of 4.6 x 10^6 $TCID_{50}$. The minimum detection limit was 1.44 $TCID_{50}$ with a corresponding Ct of 37.2. The PCR efficiency was 104.8%. C_t: threshold cycle.



SECTION C: IMPROVED DIAGNOSIS

CHAPTER 2

EFFECT OF TISSUE TYPE ON THE DETECTION OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV-IVb) BY rRT-PCR

Viral hemorrhagic septicemia virus (VHSV) is a significant threat to farm-raised and wild fish populations worldwide. Early detection of VHSV-positive populations is essential to rapidly respond to outbreaks and implement effective management strategies.

To that end, ultrasensitive and rapid real-time reverse transcription PCR (rRT-PCR) assays have been developed. Here, we evaluated the potential for sample-induced inhibition of rRT-PCR from common diagnostic samples, including combined kidney and spleen (kidney/spleen), entire viscera, and ovarian fluid. Two assays were selected based on a larger inter-laboratory rRT-PCR validation study, including Assay 1: Jonstrup et al. 2012 and Assay 2: Phelps et al. 2012. The detection of high, medium, and low amounts of VHSV-IVb was not affected with any tissue type when Assay 1 was used. However, for Assay 2, the sensitivity of VHSV-IVb detection decreased for the kidney/spleen samples spiked with low virus levels and increased for the ovarian fluid spiked with medium amounts of virus. Entire viscera, the tissue type most likely to inhibit the rRT-PCR reaction, did not affect the sensitivity of virus detection for either assay.

2.1 Introduction

Viral hemorrhagic septicemia virus (VHSV) is an important pathogen of fish, infecting a broad host range (OIE 2009). The recent emergence of VHSV-IVb in the

Laurentian Great Lakes resulted in widespread fish mortality events and regulatory responses (Lumsden et al. 2007, USDA 2009). Clinical symptoms are highly variable between host species; however, dermal and periocular hemorrhage, lethargy, and abnormal swimming behavior are common (Kim and Faisal 2011). The virus initially targets endothelial cells of the kidney and spleen, one to four days post infection. As the disease progresses, the virus spreads to other organs and tissues via the blood stream. Subsequent necrosis and degeneration of tissue can lead to organ failure and eventual death (Kim and Faisal 2011).

Early detection is essential to identify positive populations and limit further spread of the virus. To that end, ultrasensitive and rapid real-time reverse transcription PCR (rRT-PCR) assays have been developed to detect some or all strains of VHSV (Hope et al. 2010, Garver et al. 2011, Jonstrup et al. 2012, Phelps et al. 2012). The value of these assays has been demonstrated in several surveillance studies targeting the virus in the Great Lakes region (Bain et al. 2010, Cornwell et al. 2012, Phelps et al. 2012). However, the surveyed fish in these studies varied greatly in their size, and consequently, the tissue available for diagnostic testing. The recommended tissues for testing are kidney and spleen from large fish (> 6 cm), entire viscera from 3 – 6 cm fish, whole body from small fish (< 3 cm), and ovarian fluid from spawning fish (USFWS and AFS-FHS 2010). Similar recommendations are made by the International Organization for Animal Health, with the addition of heart and encephalon for fish larger than 6 cm (OIE 2009). While the sampling recommendations were established for virus isolation prior to the

development and widespread use of molecular diagnostics, they do identify a limitation of testing diverse fish populations, regardless of the technique.

Sample-induced inhibition is particularly problematic for diagnostic testing by rRT-PCR given the notoriously delicate structure of nucleic acid and enzyme sensitivity during the reaction (Loge et al. 2002, Kontani and Reed 2006, Purcell et al. 2011). Inhibitory compounds can lead to false-negative and inaccurate quantitative results (Loge et al. 2002, Guescini et al. 2008, Purcell et al. 2011). In fish, known inhibitory factors such as bile salts and urea are present in viscera and whole body samples. In addition, commonly used egg de-adhesion chemicals (tannic acid at 400 mg/L) can significantly inhibit VHSV detection by rRT-PCR (Groocock et al. 2013). It is imperative that assays are validated to account for these variables (OIE 2009, Purcell et al. 2011). Furthermore, fish health managers must understand these potential effects on test performance to accurately interpret rRT-PCR results. In this study, we examined the effects of various tissue types on the detection of VHSV-IVb by two rRT-PCR assays in fish, Jonstrup et al. (2012) and Phelps et al. (2012).

2.2 Methods

2.2.1 Tissue Collection and Preparation. To determine the effect of tissue type on the detection of VHSV by rRT-PCR, fish were selected to represent the diversity of tissue available for testing. Fish were collected live as part of an ongoing VHSV survey and immediately placed on ice for overnight transportation to the laboratory. All fish were inspected and determined negative for VHSV by virus isolation and rRT-PCR

(Phelps et al. 2012). The tissue harvested from each fish was based on recommendations of the USFWS and AFS-FHS Blue Book (2010). Kidney and spleen (2.0 g) were harvested from muskellunge, *Esox masquinongy*, larger than 6 cm in length. Entire visceral tissue (2.0 g) was harvested from 3-6 cm spottail shiners, *Notropis hudsonius*. Ovarian fluid (1.0 mL) was collected from walleye, *Sander vitreus*, broodstock. No fish less than 3 cm were available at the time of this study for whole body comparison. The negative control (no tissue) consisted of 1.0 mL of Hank's buffered salt solution (HBSS). Six replicate sets of each sample type were prepared and stored in ultralow freezer for later disbursement to six participating aquatic diagnostic laboratories. The six participating laboratories included the University of Arkansas at Pine Bluff, South Dakota State University, Cornell University, Ohio Department of Agriculture, Michigan State University, and Kennebec River Biosciences.

The tissue samples were processed according to methods recommended by the USFWS and AFS-FHS Blue Book (2010). For kidney/spleen and entire viscera, a 10% suspension (2.0 g : 20 mL) was prepared in HBSS using a stomacher at high speed for 30 sec. The homogenate was centrifuged at 4°C for 15 min at 2,900 x g. The supernatant was decanted and mixed with an equal amount (4.0 mL) of antibiotic incubation medium. The mixture was vortexed, incubated for 2 h at 15 °C, and then finally re-centrifuged for 15 min at 2,900 x g. For the ovarian fluid and the negative tissue control, 4.0 mL of antibiotic incubation media was added to the 1.0 mL of sample. Four 1.0 mL aliquots of each supernatant were prepared, for a total of 96 samples (4 tissue types x 6 sets x 4 aliquots = 96 samples).

2.2.2 Spiking tissues with virus. The virus used to spike the various tissue types was VHSV-IVb (Great Lakes isolate: MI03, Elsayed et al. 2006) grown in *epithelioma papulosum cyprini* cells (Fijan et al. 1983, Winton et al. 2010). The viral stock was diluted three times in HBSS, resulting in high (4.6×10^6 $TCID_{50}$), medium (4.6×10^4 $TCID_{50}$), and low (4.6×10^2 $TCID_{50}$) concentrations. HBSS without virus served as a negative control. The four aliquots (1.0 mL each) from the previously prepared tissue types were prepared and spiked with high, medium, or low virus concentration along with a no-virus control. For the kidney/spleen and entire viscera samples, 250 µl of the virus dilution was added to achieve a final dilution of virus to tissue suspension of 1:80. For the ovarian fluid and no tissue control, 62.5 µl of the virus dilution was added to achieve a final dilution of 1:80. All spiked samples were thoroughly mixed and immediately stored at -80°C. Sets of blinded samples were sent overnight on dry ice to each of the participating laboratories for testing by rRT-PCR. In summary, each laboratory received 16 blinded samples, consisting of the four tissue types (kidney/spleen, entire viscera, ovarian fluid, no tissue control), each with a different concentration of spiked virus (high, medium, low, no virus control).

2.2.3 Real-time reverse transcriptase polymerase chain reaction. Upon receipt, samples were immediately processed or stored at -80 °C for later testing. The RNA was extracted from each sample using the MagMax-96 Viral RNA Isolation Kit (AM1836; Life Technologies, Grand Island, NY) using either manual or robotic high throughput

processing. The extraction was performed following the Life Technologies Protocol AM1836 DW 50 v2 Aqua with a starting volume of 50 µl. The resulting RNA was held on ice for immediate testing or at -80°C for later testing. The PCR platform varied between participating laboratories and included an ABI 7500 (Life Technologies) or BioRad iCycler iQ (BioRad, Hercules, CA). Each sample was tested in triplicate with two different rRT-PCR assays at each participating laboratory, for a total of 18 replicated samples (1 sample x 6 laboratories x 3 replicates = 18 replicated samples).

2.2.3.1 Assay 1 – This assay is described in more detail by Jonstrup et al. (2012). Briefly, the 25 µl reaction mixture consisted of 12.5 µl 2X Quantitect RT-PCR Buffer (Qiagen), 0.25 µl Quantitect RT-PCR Enzyme (Qiagen), 0.225 µl of 10 µM FAM labeled TaqMan probe (5' 6-FAM-TAG AGG GCC TTG GTG ATC TTC TG-BHQ1), 0.225 µl of each 100 µM forward (5'AAA CTC GCA GGA TGT GTG CGT CC-3') and reverse (5'-TCT GCG ATC TCA GTC AGG ATG AA-3') primers, 6.175 µl nuclease-free water, and 5.0 µl of the RNA sample. The thermal cycling protocol consisted of 30 min at 50°C, 15 min at 95°C, followed by 40 cycles of 15 sec at 94°C, 40 sec at 60°C, and 20 sec at 72°C.

2.2.3.2 Assay 2 – This assay is described in more detail by Phelps et al. (2012). Briefly, the 25 µl reaction mixture consisted of 12.5 µl 2X Path-ID Multiplex RT-PCR buffer (Life Technologies), 1.25 µl of 10X Path-ID Multiplex enzyme mix (Life Technologies), 1.0 µl of 5 µM FAM labeled – MGB Probe (5'-Fam-TAC GCC ATC ATG ATG AGT- 3'), 0.375 µl of each 40 µM forward (5'-ATG AGG CAG GTG TCG GAG G-3') and reverse (5'-TGT AGT AGG ACT CTC CCA GCA TCC-3') primers

(Garver et al. 2010), 4.5 µl of nuclease-free water, and 5.0 µl of RNA sample. The thermal cycling protocol consisted of 10 min at 45°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C.

Appropriate controls were used, including negative and positive control for both extraction and amplification reactions. The threshold cycle (Ct) was determined based on automatic settings of the PCR software. All negative results were assigned a Ct value of 40. A Student's T-test was used, with a significance of $\alpha = 0.05$, to distinguish potential variation between the detection limit of VHSV in the no tissue control compared to various tissue types.

2.3 Results

Assay 1 accurately detected spiked VHSV in all tissue types at high and medium concentrations. Low concentrations of spiked VHSV resulted in positive detection of 83% (15/18) of kidney/spleen, ovarian fluid, and no tissue control samples, and 13/18 of entire viscera samples. The no virus negative control was accurate across tissue types, however the no virus-no tissue was positive in 2/3 and 3/3 replicated samples in two laboratories (Table 1).

Assay 2 accurately detected spiked VHSV in all tissue types at high virus concentration. Medium concentrations were also accurate, except for two laboratories that were not able to detect the spiked virus in the kidney/spleen samples. Detection was limited for low concentrations, with positive detection in 22% of kidney/spleen samples, 72% of entire viscera, 67% of ovarian fluid samples, and 83% of no tissue samples. The

no virus negative control was accurate across tissue types, however the no tissue sample was positive in 1/3 samples from one laboratory (Table 1).

The limit of detection among various tissue types and the no tissue control was not significantly different for Assay 1. Using Assay 2, the limit of detection was significantly different at the medium virus concentration for the ovarian fluid ($p = 0.048$) and at the low virus concentration for the kidney/spleen ($p = 0.043$) as compared to the no tissue control.

2.4 Discussion

The use of rapid and ultrasensitive methods for the detection of VHSV is important to effectively manage the disease in wild and farmed-fish populations. Consequently, the development and use of rRT-PCR for widespread surveillance has increased in recent years. Although a variety of tissue types are available for VHSV surveillance of wild and farmed-fish populations, not all have been validated for each assay despite the potential risk of PCR inhibition. In this study, the detection of VHSV-IVb using Assay 1 was not affected by tissue type, including kidney/spleen, entire viscera, and ovarian fluid. Assay 1 performed well for spiked samples considering not only the various tissue types, but the diversity of platforms and technicians across the six participating laboratories. However, five out of six no virus-no tissue negative control samples from two laboratories were weakly positive (mean Ct = 37.1), suggesting contamination of the samples at the respective or originating laboratories. The PCR products were not sequenced to evaluate non-target amplification; however, based on a

thorough validation by Jonstrup et al. (2012), Garver et al. (2011), Phelps et al. (2012), and a subsequent study by Warg et al. (personal communication) non-target amplification is unlikely. Regardless, these results highlight the importance of appropriate controls and caution interpreting rRT-PCR results.

The detection of VHSV-IVb using Assay 2 was reduced in the kidney/spleen samples spiked with low levels of virus, with only 22% of the samples being positive. However, virus detection was significantly improved for ovarian fluid samples spiked with medium levels of virus. Entire viscera, the tissue type most likely to inhibit the rRT-PCR reaction, did not affect the sensitivity of virus detection. Given these inconsistent results, it is more likely an indication of the robustness of the assay across platforms, not inhibition or promotion by any tissue type. This conclusion is supported by Warg et al. (personal communication), where similar results were observed for Assay 2. Assay refinement with additional validation is needed on various platforms to ensure consistency prior to the widespread use of Assay 2.

This study standardized viral levels between the tissue types, however viral pathogenesis must be considered. The selection of tissue for rRT-PCR should remain based on sites with high viral replication, such as kidney and spleen. However, in the event these tissues are not available in sufficient quantity, this study has shown the detection of low viral levels was not affected by the entire viscera samples. The inhibitory potential on various strains of VHSV or from various host species should also be evaluated. Performing similar studies on experimentally or naturally infected fish would be valuable.

Although this study has demonstrated the inhibitory effects of a diverse sample set may be negligible for the two evaluated assays, continued evaluation must be performed. This can be achieved by the incorporation of internal positive controls. Numerous sources recommend the routine use of internal positive controls to monitor for potential inhibition of rRT-PCR assays; however, this is not yet common practice in veterinary diagnostics (Matejusova et al. 2008, Bustin et al. 2009, Purcell et al. 2011). While additional validation would be needed for many of the commonly used assays, this effort would significantly increase the confidence in diagnostic results and in turn, the health of animal populations.

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Table 2.1. Detection of viral hemorrhagic septicemia virus-IVb by real-time reverse transcriptase PCR for three tissue types using two assays. Samples were spiked with high, medium, or low virus dilutions, or no virus control. * indicates Ct values significantly different than the no tissue control. Ct = Cycle threshold. Sd = Standard deviation. % Pos = Percent positive.

Tissue	Kidney/Spleen		Viscera		Ovarian Fluid		No Tissue	
Assay	1	2	1	2	1	2	1	2
High								
Ct	20.35	19.54	23.03	22.94	19.98	20.22	19.58	20.13
Sd	1.80	0.98	4.00	2.66	1.89	2.09	1.80	1.88
% Pos	100	100	100	100	100	100	100	100
Medium								
Ct	27.61	30.47	28.33	27.23	27.23	26.26*	27.92	27.32
Sd	1.74	7.43	2.36	0.87	1.70	1.04	1.07	0.50
% Pos	100	67	100	100	100	100	100	100
Low								
Ct	35.28	38.47*	36.14	35.36	35.42	34.50	35.25	34.18
Sd	2.75	3.44	3.22	3.46	2.58	4.39	2.61	2.98
% Pos	83	22	72	72	83	67	83	83
No virus								
Ct	40.00	40.00	40.00	40.00	40.00	40.00	39.76	39.26
Sd	0.00	0.00	0.00	0.00	0.00	0.00	0.60	1.39
% Pos	0	0	0	0	0	0	28	6

SECTION D: IMPROVED MANAGEMENT

CHAPTER 3

THE IMPORTANCE AND METHODS USED IN A FISH HEALTH INSPECTION

Published as:

Fish Health Collection Manual, 2012 Edition

The emergence of viral hemorrhagic septicemia virus (VHSV-IVb) in fish in the Great Lakes region has resulted in a significant regulatory response to better manage the disease. Prior to the movement of VHSV-susceptible fish in Minnesota, the originating waterbody must be certified VHSV-free within the past year. Furthermore, in 2009 Minnesota enacted legislation requiring that fish for regulatory health inspections must be collected by a 'Fish Health Collector' approved for that job by the Minnesota Department of Natural Resources (MNDNR). To be approved as a Fish Health Collector an individual was required to be a 1) American Fisheries Society – Fish Health Section Fish Health Inspector or Pathologist, 2) Accredited veterinarian with approved training, and 3) other individual (i.e. MNDNR field biologist) with approved training. In response to this legislation, a fish health collector training workshop was developed for veterinarians and field biologists to fulfill the MNDNR requirements. The resulting manual (given below) is intended to supplement this full-day workshop and provide the basic information and references to perform a fish health collection. This manual is not intended to be a diagnostic or regulatory guide, as these fields change frequently. The manual should be regularly updated and available through the MNDNR to reflect the current fish health collector requirements in Minnesota and elsewhere.

3.1 Introduction

The need for a cooperative approach to managing the health of wild and farmed fish populations is more important than ever, as disease, environmental, and public health concerns continue to grow. These concerns are complicated by many factors, including increasing interconnectedness of our waters and animals, rapid growth of the aquaculture industry, and the lack of trained professionals. This need has been highlighted with the recent emergence of viral hemorrhagic septicemia virus (VHSV-IVb) in the Great Lakes region. While VHSV is not the first major fish disease in North America, it has resulted in unprecedented regulatory and biological concern, requiring increased efforts to improve fish health management.

One of these efforts, and the purpose of this manual, is to prepare veterinarians and fisheries biologists to perform fish health sample collections associated with disease surveillance and monitoring in the state. The lack of trained professionals to perform fish health collections is often cited as a major bottleneck to satisfying regulatory requirements. In addition to serving as an essential link between the field and diagnostic laboratory during emergency events, these fish health collectors will be available to benefit the fish health needs of the industry and natural resource agencies.

In Minnesota, a fish health collector must attend a Minnesota Department of Natural Resources (MNDNR)-approved training course. The intention of this manual is to supplement this course and provide a reference while in the field for certified Minnesota Fish Health Collectors. This is not meant to be a diagnostic or regulatory

manual, as both of these areas change frequently. It will however, outline the necessary steps to perform a fish health collection in Minnesota. While the majority of this manual could be applied in other States, variations do exist and should be considered prior to sample collection.

3.2 Federal and State Regulations

State and federal regulations are highly variable and can change frequently, often without notice. The following regulations are not a thorough explanation of all rules pertaining to aquaculture, but rather those that may be referred to for a fish health collection. Competent authorizes must be consulted prior to collecting fish for up-to-date and pertinent regulations.

3.2.1 Federal Fish Health Regulations

In 2006, USDA imposed strict movement restrictions on fish in the Great Lakes region to limit the spread of VHSV and to protect valuable aquaculture industries. In 2008, the USDA amended their Federal Order for VHS to its current language (USDA-APHIS 2008). The Amended Federal Order prohibits or restricts interstate movement of VHSV-susceptible fish species for affected or at-risk states (IL, ID, MI, MN, NY, OH, PA, and WI) and provinces (Ontario, Quebec). The following summarizes the 2008 Amended Federal Order.

1. VHSV-susceptible species included under the Amended Federal Order, are those found positive for VHSV-IVb by virus isolation under natural (i.e. non-experimental) conditions in the United States and Canada.

Black crappie *Pomoxis nigromaculatus*	Bluegill *Lepomis macrochirus*
Bluntnose minnow *Pimephales notatus*	Brown bullhead *Ictalurus nebulosus*
Brown trout *Salmo trutta*	Burbot *Lota lota*
Channel catfish *Ictalurus punctatus*	Chinook salmon *Oncorhynchus tshawytscha*
Emerald shiner *Notropis atherinoides*	Freshwater drum *Aplodinotus grunniens*
Gizzard shad *Dorosoma cepedianum*	Lake whitefish *Coregonus clupeaformis*
Largemouth bass *Micropterus salmoides*	Muskellunge *Esox masquinongy*
Shorthead redhorse *Moxostoma macrolepidotum*	Northern Pike *Esox lucius*
Pumpkinseed *Lepomis gibbosus*	Rainbow trout *Onchorhynchus mykiss*
Rock bass *Ambloplites rupestris*	Round goby *Neogobius melanostomus*
Silver redhorse *Moxostoma anisurum*	Smallmouth bass *Micropterus dolomieu*
Spottail shiner *Notropis hudsonius*	Trout-Perch *Percopsis omiscomaycus*
Walleye *Sander vitreus*	White bass *Morone chrysops*
White perch *Morone Americana*	Yellow perch *Perca flavescens*

2. Permissible international movement of live VHSV-susceptible species:
    a. Salmonids imported into the United States must meet requirements established in Title 50, Code of Federal Regulations, Sections 16.13 (a) (3) and 16.13 (b).
    b. Non-salmonids may be imported for direct slaughter with prior USDA-APHIS approval.

3. Permissible interstate movement of live VHSV-susceptible species:
    a. Destined to slaughter:
        i. May be exempt from requirements with a USDA-APHIS-VS Form 1-27.
    b. Destined to diagnostic or research laboratory:
        i. Fish must be transported to approved laboratory, authorized by Federal, State, or Tribal competent authority.
        ii. Fish must be accompanied by USDA-APHIS-VS Form 1-27.
    c. Destined to other locations:
        i. Fish must be certified VHSV-negative within last 12 months, according to the approved VHSV inspection methods.
4. VHSV Inspection:
    a. The inspection and sampling requirements must follow the federally accepted guidelines of the American Fisheries Society-Fish Health Section (AFS-FHS) Blue Book (USFWS and AFS-FHS 2010) or the World Organization for Animal Health (OIE) Diagnostic Manual of Aquatic Animal Diseases (OIE 2009).
    b. Collection of the fish and laboratory inspection must be performed by a certified individual, which may include a AFS-FHS fish pathologist, AFS-FHS fish health inspector, accredited veterinarian, or an individual authorized by the federal, state, or tribal authority.

5. Catch-and-release activities (i.e. tournament, recreational fishing, etc.) are allowed, except for VHSV-susceptible species used/intended as bait.

3.2.2 State Fish Health Regulations: Minnesota

In addition to the aforementioned federal order, many states have fish health requirements for inter- and intra-state transportation. Given the diversity of wild and farmed fish populations and their associated risks, the regulations are highly variable across the Great Lakes region. Tolerable risk also varies for each state and consequently, no agreeable solutions have been reached. This complex array of regulations is problematic for the industry, as well as fish health collectors trying to stay current. It is important to note that state regulations change frequently and regular contact with their competent authority (i.e. Department of Natural Resources, Department of Agriculture, etc.) is advised (NCRAC 2012). As such, this manual will focus on one state as an example: Minnesota (MNDNR 2012). For more information of Minnesota fish health requirements, please contact the Minnesota Department of Natural Resources (MNDNR): Ling Shen (651-259-5138) or Paula Phelps (651-259-5213).

3.2.2.1 VHS susceptible species

1. VHSV-susceptible species imported, transported, or stocked within Minnesota must be certified VHSV-negative within last 12 months, according to the approved VHSV inspection methods.
2. Inspection Methods:

a. Inspection and sampling must follow the federally accepted guidelines of the AFS-FHS Blue Book or the OIE Diagnostic Manual of Aquatic Animal Diseases.
    i. Exception: Wild harvest baitfish (see below).
b. Collection of fish and laboratory inspection must be performed by MNDNR certified Fish Health Collector, this includes:
    i. AFS-FHS fish pathologist or fish health inspector
    ii. Accredited veterinarian with additional MNDNR-approved training
    iii. Individual with additional MNDNR-approved training authorized by the MNDNR (i.e. MNDNR fisheries biologist).

3. Wild harvest baitfish:
    a. Due to the rapid diagnostic turnaround needed to maintain the wild baitfish industry, fish may be harvested if certified VHSV-negative by rRT-PCR. Virus isolation must also be performed to support rRT-PCR, but movement can occur prior to test completion.
        i. The approved assay has been well validated by the OIE reference laboratory (Jonstrup et al. 2012) and the USDA (Warg et al. in preparation).
4. Importation, stocking, and transportation of VHSV-susceptible species require a MNDNR live fish transportation, importation, and stocking permit.
    a. Contact: Neil Vanderbosch (651-259-5178)

b. The permit application is available at:

http://files.dnr.state.mn.us/rlp/permits/fishery/livefish_transport.pdf

5. Reporting:
    a. Laboratory results must be reported to the MNDNR when available to receive transportation permit.
    b. In addition to VHSV, all replicating viruses identified by routine virus isolation must be reported (only applies to minnows imported for later export or feeding hatchery fish under a MNDNR permit).

3.2.2.2 Salmonids

1. Salmonid species imported, transported, or stocked within Minnesota must be certified negative for VHSV, infectious hematopoietic necrosis virus, whirling disease, *Yersinia ruckerii*, *Renibacterium salmoninarum*, and *Aeromonas salmonicida* within the last 12 months, according to the approved inspection methods.
2. Inspection Methods:
    a. Inspection and sampling must follow the federally accepted guidelines of the AFS-FHS Blue Book or the OIE Diagnostic Manual of Aquatic Animal Diseases.
    b. Collection of fish and laboratory inspection must be performed by MNDNR certified Fish Health Collector, this includes:
        i. AFS-FHS fish pathologist or fish health inspector

ii. Accredited veterinarian with additional MNDNR-approved training

iii. Individual with additional MNDNR-approved training authorized by the MNDNR (i.e. MNDNR fisheries biologist).

3. Importation, stocking, and transportation of salmonids requires a MNDNR live fish transportation, importation, and stocking permit.
    a. Contact: Neil Vanderbosch (651-259-5178)
    b. The permit application is available at: http://files.dnr.state.mn.us/rlp/permits/fishery/livefish_transport.pdf
4. Reporting:
    a. Laboratory results must be reported to the MNDNR when available to receive transportation permit.

3.2.2.3 Catfish

1. Catfish species imported, transported, or stocked intra-state must be certified negative for VHSV, Channel catfish virus, and *Edwardsiella ictaluri* within the last 12 months, according to the approved inspection methods.
2. Inspection Methods:
    a. Inspection and sampling must follow the federally accepted guidelines of the AFS-FHS Blue Book or the OIE Diagnostic Manual of Aquatic Animal Diseases.

b. Collection of fish and laboratory inspection must be performed by MNDNR certified Fish Health Collector, this includes:
    i. AFS-FHS fish pathologist or fish health inspector
    ii. Accredited veterinarian with additional MNDNR-approved training
    iii. Individual with additional MNDNR-approved training authorized by the MNDNR (i.e. MNDNR fisheries biologist).

3. Importation, stocking, and transportation of catfish requires a MNDNR live fish transportation, importation, and stocking permit.
    a. Contact: Neil Vanderbosch (651-259-5178)
    b. The permit application is available at: http://files.dnr.state.mn.us/rlp/permits/fishery/livefish_transport.pdf
4. Reporting:
    a. Laboratory results must be reported to the MNDNR when available to receive transportation permit.

3.3 Performing a Fish Health Collection

3.3.1 Scheduling and Forms:

It may seem obvious that the fish health collection must be scheduled with the farm and diagnostic laboratory and appropriate paperwork must be completed in a timely fashion, however far too often these tasks are not performed. This section is intended to

serve as a reminder: to receive timely results, scheduling and completing the necessary paperwork is required.

3.3.1.1 Scheduling the fish health collection:

Collecting fish is often unpredictable and requires a high degree of flexibility. When the owner calls to schedule a fish health collection, be sure the fish will be available and the diagnostic laboratory has been contacted and is prepared to receive the submission. This will save significant time in the field and avoid problems at the laboratory. Typically, diagnostic laboratories require the fish to arrive Monday – Thursday to allow sufficient processing time before the weekend. Delayed or surprise deliveries can result in postponed testing, which may require re-submission of the fish. Thus, you should always notify the diagnostic laboratory that you are planning a collection and when you have shipped the samples.

There are many well qualified diagnostic laboratories that service the aquaculture industry in the United States. Selection of the laboratory is based on owner/collector preference. Minnesota is fortunate to have two local laboratories, including:

University of Minnesota
Veterinary Diagnostic Laboratory
Attn: Nick Phelps
612-624-7450
phelp083@umn.edu
http://www.vdl.umn.edu/ourservices/fish

Minnesota Department of Natural Resources
Pathology Laboratory
Attn: Ling Shen
651-259-5138
ling.shen@state.mn.us
http://www.dnr.state.mn.us/eco/pathlab

3.3.1.2 Necessary forms and paperwork

Thorough record keeping is an essential part of a fish health collection. While it may be advantageous to keep more intensive records on file, at minimum, the sample submission form for the appropriate diagnostic laboratory must be completed. The information provided on the sample submission form will be directly transferred to the eventual fish health report/certification. Incomplete forms or errors will result in delays, confusion, and potentially lost results. Special attention should be paid to include the owner and collector information, collection location (appropriately identified), the type of sample submitted, and the requested tests. Sample submission forms should always be completed at the time of collection, when the owner is present, to ensure that information provided is accurate and complete. Make sure to include a complete copy of the sample submission form, in a sealed plastic bag, along with the sample to be shipped to the diagnostic laboratory. If you have questions pertaining to completing a sample submission form, please be sure to contact the appropriate diagnostic laboratory prior to collection.

3.3.1.3 Requesting the appropriate tests

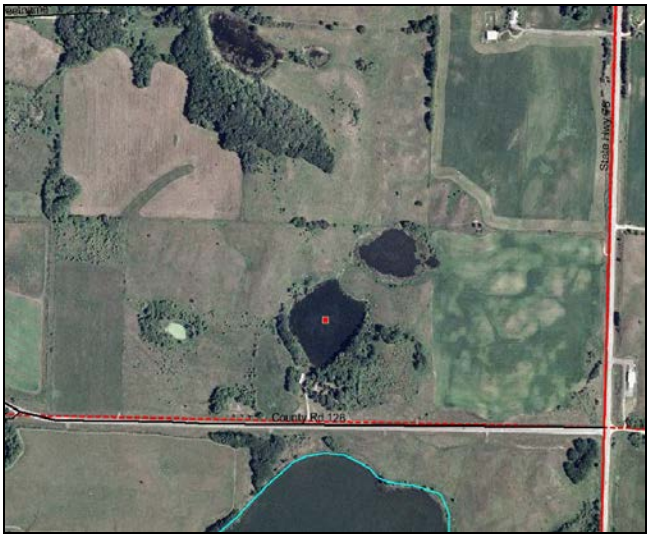
The fish health collector and owner must decide which tests to request. This will be determined based on the regulatory requirements of the receiving state and the species of the fish. The fish health collector should inform the diagnostic laboratory which tests are being requested both when scheduling and on the sample submission form. Failure to request the necessary tests will result in delayed results or require resubmission of samples.

If you are not confident in your knowledge of the current regulations, contact the fish health official for the receiving state. Regulations change frequently and are highly variable between jurisdictions. See the Federal and State Regulation section for more information. You will need to know how many fish should be collected and what tests are required.

3.3.2 Waterbody Identification

The primary purpose of the fish health collector is to maintain the chain of custody for the sample from the source waterbody to the diagnostic laboratory. Therefore, it is essential to accurately identify the location from which the fish originated. While this seems self-evident, it can be challenging. Many ponds used for fish production and streams used for wild baitfish harvest are remote, often with multiple waterbodies of the same size in close proximity, and small waterbodies are frequently omitted from maps. To further complicate the process, one waterbody may be known by several names or identification numbers, depending on the source of information. When in doubt, the more information included, the easier it will be to identify the source by the regulatory agency.

Figure 3.1. Typical waterbody used for walleye or baitfish production by the aquaculture industry in Minnesota.



3.3.2.1 Commonly used methods for identification:

Common name: Many waterbody common names are readily accessible and serve as the primary identification method. However, small waterbodies used by the aquaculture industry have no standardized name and are often referred to by the name of the property owner (i.e. Johnson Pond). While appropriate for local reference, it can be confusing or impossible for regulatory agencies to locate these ponds. In addition, certain lake names are used frequently (i.e. Mud Lake, Green Lake, etc). While the common name should be provided, an additional identification method should also be included.

Global Positioning System: GPS coordinates are the ideal identification method for waterbodies in remote locations or specific points on larger waterbodies. Over time these numbers will not change and are widely accepted by regulatory agencies. In addition, there is little room for doubt in the field, and no need to trust the owner to provide

accurate information or try to locate the waterbody on a map. If possible, this method of identification should always be used.

Public waters inventory number: Identification numbers are often assigned to waterbodies by natural resource management agencies. In Minnesota, the MNDNR uses a Department of Waters (DOW) number or Kittle number to identify waters of the state, including many of the waters used by the aquaculture and baitfish harvest industries. The DOW or kittle number is linked to a central MNDNR database, therefore access is limited and confirmation in the field can be difficult. In addition, not every waterbody has been assigned a DOW or kittle number at this point in time. If you are unsure of the correct DOW or kittle number, you should take GPS coordinates.

Public Land Survey System: The use of Township – Range – Section as a method to identify waterbodies is common. Like the use of GPS, these coordinates are widely recognized and accepted. However, if you are working in a location with many waterbodies of similar size in close proximity this method can be problematic. In this case, you should not use this method to identify the source location as confirmation by the regulatory agency will be difficult.

Aquaculture facility: Aquaculture facilities pose an identification challenge. These locations are typically composed of multiple ponds in very close proximity. In this case, each pond should be identified by the facility license number and specific pond number. The information should be connected back to their current aquaculture license held by both the farm manger and the regulatory agency. It is important to note that over time the facility and associated identification scheme can change, thus you need to ensure that you

are looking at the most current license. As with any identification method, the accuracy of the information should be regularly verified with a secondary source and owner.

3.3.3 Fish Identification and Anatomy

During the course of the fish health collection, the collector must record the species(s) of interest and incidental species on the submission form. It is advised that a fish health collector become familiar with the common fish species of Minnesota. This process is relatively straight forward for the mono-cultured species with few look-alikes, but may be more challenging for baitfish harvested from the wild. Species of particular importance for the fish health collection are those susceptible to VHSV. The following images highlight the four most frequently collected fish species for a regulatory inspection:

Figure 3.2. Spottail shiners are a small to medium sized minnow, with adult sizes averaging 3.5 inches in length. The defining feature of the spottail shiner is a distinct black spot at the base of the caudal fin. The coloration can range from silvery to pale green to olive color. Spottail shiners are commonly harvested from creeks in Central Minnesota from May to June.

Figure 3.3. Emerald shiners are small to medium sized minnows, with adult sizes averaging 3.5 inches in length. The coloration is a bright silvery to emerald appearance. Emerald shiners are commonly harvested from rivers and lakes in Northern Minnesota (near Canadian border) from October to November.

Figure 3.4. The walleye is the state fish of Minnesota and the most popular farm raised species. Walleye spawn in the spring and are typically harvested in outdoor ponds September to October for stock enhancement. The size at harvest can vary from 4-6 inches, with carryover fish and brood stock growing much larger. Walleye are characterized by dark green to gold coloration, with a white spot on the caudal fin.

Figure 3.5. Yellow perch is a popular sportfish in Minnesota, most commonly farm raised for stock enhancement; however there is a growing food market as well. The yellow perch is often identified by the dark vertical bars and gold to yellow coloration. Yellow perch spawn in the spring and grow to approximately 4 inches by October in

outdoor ponds. In indoor systems yellow perch can reach food market size (8-10 inches) in 10-14 months.

More information on fish identification can be found at:

http://infotrek.er.usgs.gov/wdnr_fishes/index.jsp

In addition to species identification, the fish health collector must have a basic understanding of fish anatomy. This is essential in performing the fish health assessment, as well as dissecting specific tissues (if required) for diagnostic testing. The following images highlight the common characteristics of fish (both farm raised and wild caught) in Minnesota.

Figure 3.6. External anatomy of a bluegill.

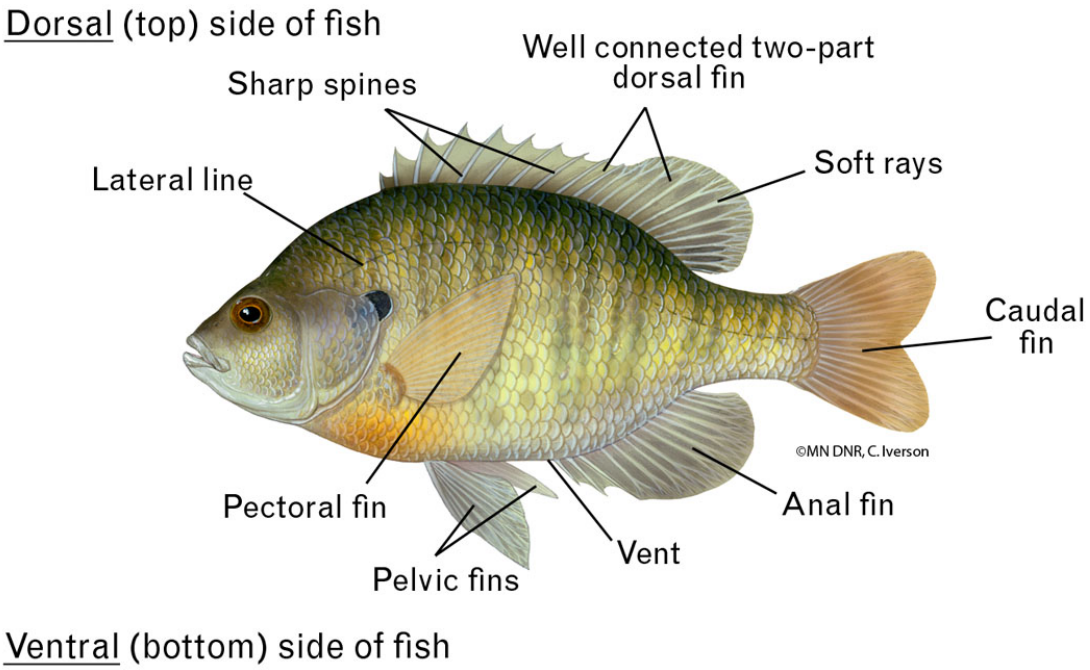
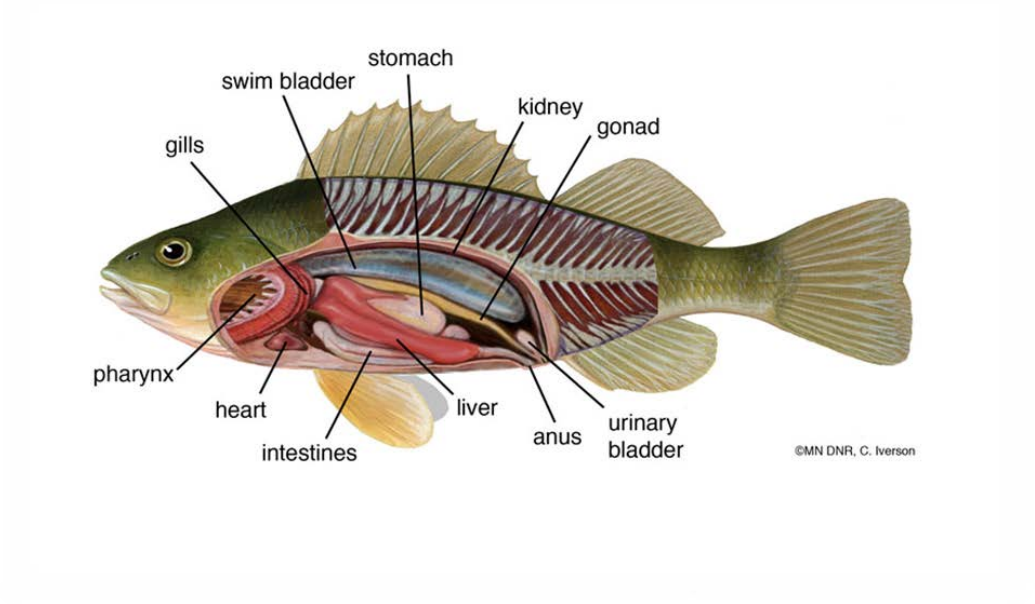


Figure 3.7. Internal anatomy of a yellow perch.



3.3.4. Fish Health Assessment

During the fish health collection, it is advised to take note of the general condition of the fish population. This so-called “fish health assessment” is an initial observation that can be very thorough, with a detailed assessment of a wide variety of metrics, including signs of disease, body fat, organ condition, etc.. This level of attention can be valuable to a fish producer or natural resource manager to monitor condition trends in the population and serve as an early indicator of disease. However, for the purpose of a typical fish health collection, the fish health assessment need not be as detailed. At a minimum, a few sentences about the external appearance should be included. In some cases, a statement of the fish health assessment is required for the transportation certificate, making accurate observations critical. While the fish health assessment can be performed at the diagnostic laboratory, post mortem changes do occur during transit that can significantly influence the assessment.

It is important to note that a diagnosis cannot be achieved based on visual inspection alone. Similar lesions can result from a wide variety of pathogens. Therefore, as the fish health collector, understanding “normal” and describing “abnormal” is the primary purpose of the fish health assessment. It is essential to become familiar with the population of interest. Included here are examples, albeit extreme, of clinical lesions in fish. They are intended to provide clinical descriptions for a fish health assessment, not a diagnosis of a particular pathogen.

Figure 3.8. The bluegill on the left appears normal, with no apparent sign of contagious or infectious disease. The bluegill on the right has severe focal hemorrhage in the eye

and operculum. Diffuse or ecchymotic hemorrhage (bruising) can be seen on lateral sides along with moderate to severe erosion of the caudal and ventral fins. Slight emaciation is also observed.

Figure 3.9. Northern pike with severe scale loss and ulcers at the caudal peduncle. The ulcer has clean margins, is approximately 4 cm round, and penetrates into the skeletal muscle.

Figure 3.10. Goldfish with an ulcer centered on the lateral line. The ulcer is poorly demarcated, with bloody margins approximately 2.5 cm by 4.0 cm. The majority of the ulcer is limited with only scale loss, while portions penetrate into the skeletal muscle.

Figure 3.11. Koi with moderate to severe necrosis of the gill. Necrosis resulted in light brown appearance of the filaments.

Figure 3.12. Rainbow trout with varying degrees of skeletal abnormalities: Top fish appears normal, with no clinical signs of contagious of infectious disease. Middle fish has moderate scoliosis and discoloration on caudal half of body. Third fish is in poor condition, with severe scoliosis at caudal peduncle, complete erosion of caudal fin, and moderate erosion of operculum exposing the gills.

Figure 3.13. Pumpkinseed with diffuse hemorrhage throughout the fish, including mouth, operculum, fins, and skin. Focal hemorrhage at base of fins and vent.

Figure 3.14. Bluegill with severe infection of diegetic trematodes (“black grubs”) encysted in the skin.

3.3.5 Sample Collection

The methods used to collect fish are as diverse as the species and systems from which they come. It is the responsibility of the fish health collector to verify the fish were collected from the specified waterbody and enter this information on the submission form.

It is essential to verify that the owner has randomly selected the fish from the population. This can be done by trap, seine, electroshocking, etc. Targeting fish that are feeding could bias the sample to healthy individuals. One exception is to target fish that appear unhealthy. According to the AFS-FHS Blue Book, one sick fish (with clinical signs of a reportable pathogen) can represent five healthy fish in the sampling protocol. Equipment and tools should not be shared between different lots without proper disinfection to eliminate sample contamination.

3.3.5.1 Number of fish to be collected

The number of fish collected depends on the type of production system and the regulations of the receiving state. There are two primary considerations that must be made to ensure the appropriate samples are collected.

Farm vs Lot inspection: Lot inspections test isolated or unique units of fish based on location, age, and species that share the same water source. The water source may be a well, pond, stream, etc. Due to the homogeneity of a lot, fewer fish (typically 60) are needed per lot because one could assume the pathogen, if present, is more likely to be found. This approach is advantageous for locations that cannot maintain biosecurity or

when lots can be tested on an as-needed basis. In addition, this approach is useful for isolated locations (i.e. a single walleye pond) where fewer fish would need to be tested.

A farm (or facility) level inspection is very different from a lot inspection. Briefly, performing a fish health collection on an entire facility with a single water source requires all fish (or lots) to be proportionally inspected. For example, if there are 10 ponds with equal numbers of fish in each and 150 fish must be collected, the collector would obtain 15 randomly selected fish from each of the 10 ponds. This assumes the facility is one unit. In the event one pond is identified as positive for a particular pathogen, all are considered positive. Furthermore, biosecurity must be maintained for the entire facility following the inspection to maintain confidence in the results. A breach in this biosecurity (i.e. introduction of new fish) for any pond would effectively void the diagnostic results.

Number of fish to collect: The sample size is determined based on two factors: pathogen prevalence and desired confidence. The assumed pathogen prevalence level (APPL) is a highly variable value based on specific pathogens, unique host populations, and environmental or seasonal conditions. Therefore, assumed APPL's of 2-10% are regularly used based on the typical expression of a disease in apparently healthy populations. The desired confidence in the inspection is often estimated to be 95%. The statistical analysis used to generate the appropriate sample size also assumes that the test to identify a pathogen is perfect. While this may not be true, assurance is maintained with relatively high level of desired confidence and low assumed APPL.

Common sample sizes for fish health collections of large populations are 60 fish with a 5% APPL for lot inspections and 150 fish with a 2% APPL for farm inspections. Exceptions are made for small populations of valuable broodstock or other circumstances where smaller samples sizes are unavoidable.

3.3.5.2. Type of sample

Two types of samples are commonly submitted to diagnostic laboratories for the detection of viral agents: whole fish and dissected tissue. The selection is dependent on the fish health collector's experience and diagnostic laboratory requirements. Fish of all sizes are suitable for viral testing, following yolk sac adsorption. Ovarian fluid is a suitable sample from female broodfish. The size/age of fish should represent the total population.

Whole fish: Whole fish are commonly accepted by diagnostic laboratories; however, some will charge an additional fee for processing. The fish should be humanely euthanized and placed into a sealable plastic bag. The fish should be immediately put on frozen gel packs for shipping.

Dissected tissue: Dissecting the fish to obtain tissues may be required by some diagnostic laboratories. In some cases, this method may be preferred by fish health collectors performing a thorough fish health assessment. Care should be taken to ensure the samples are appropriately collected. If the fish health collector is not experienced in dissecting fish, they should contact the diagnostic laboratory for directions or simply

submit the whole fish. Delay or rejection of samples may occur if samples are not appropriately prepared.

The fish should be humanely euthanized prior to dissection. During processing the samples should be kept cold and at no time exceed 15°C. Fish tissue may be pooled in groups of up to five fish per sample. Aseptic methods should be used to prevent cross contamination and tissue placed in sterile containers for shipping. Four types of tissue are suitable for virus isolation, depending on the size of fish, including:

1. Fry (less than 3cm): The entire fish should be collected.
2. Fingerling (3cm – 6cm): The entire viscera, including the kidney should be collected. If the stomach/intestine is filled with food, it should be removed.
3. Yearling/Adult (greater than 6cm): Equal amounts of the kidney and spleen should be collected.
4. Ovarian fluid: At least 1 mL of fluid should be collected from each female broodfish.

3.3.6. Sample packaging and shipping

Once the fish are collected, they should immediately be sent to the diagnostic laboratory. The samples must remain cool and arrive within 24 hours of collection, therefore overnight shipping or personal delivery is required. You should always contact the diagnostic laboratory prior to shipping or delivering the fish so proper attention can be given upon their arrival.

To maintain the quality of the samples, they must remain cool for the duration of transit. If the fish are sent live, adequate oxygen should be added to the container and the water temperature must remain cool. If the fish are sent dead, place the fish in clear plastic sealable bags with sufficient frozen gel packs to remain cold (not frozen) until delivery. Wet ice should not be used for mail delivery because the melted ice commonly leaks from shipping containers.

To prevent a breach in the chain of custody, the shipping container (box, bag, cooler, etc.) must be sealed by the fish health collector. Alternatively, if they cannot seal the container, the fish health collector can ship (or accompany the owner to the shipping location) the container himself/herself. Multiple lots may be included in the same delivery, but be certain to avoid contamination between the groups of fish. The fish should also be clearly labeled with the waterbody identification number and species that correspond to the submission form. The completed submission form must be included with the samples, in a separate sealed bag.

Figure 3.15. The figure on the left shows properly packaged samples: clearly labeled, sufficient frozen gel packs, and the submission form in a separate sealable bag. The photo on the right shows improperly packaged samples: samples not labeled and no submission form.

3.3.7. Results and Reporting

Following the completion of testing at the diagnostic laboratory, the results will be provided to the owner and the fish health collector or the associated agency. Typically, these results are confidential and are not shared with others, unless specifically requested. If additional recipients are not requested on the sample submission form, it will be the responsibility of the owner or collector to distribute the results. Exceptions may include state/federally funded testing where the results are public or results from the MNDNR pathology laboratory that can be obtained by other parties if they pay a proportional share of the cost.

If the fish are destined for interstate movement, contact the receiving state's fish health official to be sure the results are submitted to the appropriate individual and in the correct format. Some states provide state-specific forms (i.e. New York and Wisconsin) that must be completed by the fish health collector and/or the diagnostic laboratory. Attention to this at the time of the collection will ensure the results are reported in a timely manner and fish movements are not delayed.

3.3.8. Summary

1. Determine the source location (waterbody, facility, etc) of the fish
2. Contact the diagnostic laboratory to confirm that the owner has scheduled testing. Fish health collections should be scheduled a minimum of one week in advance. Schedule the collection to ensure the samples arrive to the diagnostic laboratory Monday – Thursday.
3. Determine the sample size and required tests. Check with state fish health official if uncertain.
4. Gather all necessary supplies (plastic bags, shipping container with frozen gel packs, marker/pencil, GPS, submission form, etc.)
5. Once the fish are collected, bag and immediately place on frozen gel packs for shipping. Be sure to separate different lots of fish to prevent contamination. Label each lot with the waterbody name and species.
6. Complete the sample submission form at the time of collection. Ensure that you have included the owner and collector information, date, location, species, and requested tests.
7. If collecting tissue: Follow the procedures for collection of tissues for the detection of viral agents.
8. Place all samples from the same waterbody into a sealable bag. Label the bag and immediately place on frozen gel packs for shipping.

9. Place sample submission form in a sealable bag in the shipping container alongside the fish samples.
10. Samples should be shipped in a cooler with sufficient frozen gel packs to keep samples cool throughout transport. Samples must arrive at the diagnostic laboratory within 24 hours of sample collection – use overnight delivery.
11. Ship samples to the diagnostic laboratory.
12. Report results to appropriate individuals.

3.4 References

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SECTION D: IMPROVED MANAGEMENT

CHAPTER 4

COMPARISON OF TREATMENTS TO INACTIVATE VIRAL HEMORRHAGIC SEPTICEMIA (VHSV-IVb) IN FROZEN BAITFISH

Published as:

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In the USA, current state and federal fish health regulations target the spread of viral hemorrhagic septicemia virus-IVb (VHSV-IVb) through movement restrictions of live fish but largely ignore the potential for the virus to be spread through the commercial distribution and use of frozen baitfish from VHSV-IVb positive regions. Some state laws do require treatment of frozen baitfish to inactivate VHSV and additional methods have been proposed, but there have been few scientific studies examining the efficacy of these treatments. In this study, bluegills were challenged with VHSV-IVb and frozen to represent standard industry methods, disinfected by various treatments, and tested for

infectious VHSV-IVb using virus isolation. The virus was isolated from 70% of fish subjected to three freeze thaw cycles. All other treatment methods were effective in inactivating the virus, including treatment with isopropyl alcohol, mineral oil, salt and borax, and dehydration. Dehydration followed by rehydration is rapid and effective, and therefore, seems to be the best option for inactivating VHSV-IVb present in frozen baitfish while maintaining their usefulness as bait.

4.1 Introduction

Viral hemorrhagic septicemia virus (VHSV) strain IVb was first isolated from the Laurentian Great Lakes in 2003 (Elsayed et al. 2006, Lumsden et al. 2007). Since then, the virus has spread throughout the Great Lakes basin causing large mortality events in a wide variety of species. The route by which VSHV entered the Great Lakes is unknown; however, water connectivity and natural fish movement continue to distribute the virus (Thompson et al. 2011). Of particular concern has been the spread of VHSV-IVb to inland lakes, including one outside the watershed. In these cases, likely transmission

routes include water, fomites, and movements of fish for stock enhancement and bait (Bain et al. 2010, VHSV Expert Panel 2010).

The potential to inadvertently move aquatic animal pathogens to new regions through shipments of baitfish is a concern for fish health managers (Hedrick 1996, Harvell et al. 1999, Gaughan 2002, Goodwin et al. 2004). Baitfish are often used in close proximity to their source; however, larger numbers of live and frozen baitfish captured from the wild are moved and released into new regions far from their origins (Goodwin et al. 2004). Frozen baitfish are particularly concerning, given the large harvest volume, wide geographic distribution, and evidence that freezing is not sufficient to inactivate all virus present in infected fish (Meyers et al. 1999, Gaughan 2002, Arkush et al. 2006, Herve-Claude et al. 2008). In fact, freezing (-20 to -10°C) has been shown to be suitable for preservation of a variety of viruses in fish tissue (Plumb et al. 1973, Burke & Mulcahy 1983, Plumb & Zilberg 1999).

There are several well-documented examples of fish disease introductions that appear to have been caused by movements of frozen baitfish. In 1995 and 1998, outbreaks of the pilchard herpes virus (PHV) in Australia resulted in fish kills and a 70% decline of the spawning biomass of sardines, *Sardinops sagax* (also known as pilchards) (Gaughan 2002). The PHV is believed to have been exported to Australia in frozen and unprocessed sardines, that were fed to tuna raised in sea pens (Hyatt et al. 1997, Gaughan 2002). In 2004, more than 2,500 sea bass, *Dicentrarchus labrax*, died on a farm in the Mediterranean Sea. The kill was attributed to VHSV

(http://www.cefas.defra.gov.uk/idaad/abstract.aspx?t=do&id=) and speculated to have been introduced with frozen baitfish fed to a nearby tuna farm (WWF 2005).

In the USA, large volume frozen baitfish shipments originate from regions known to be VHS-IVa or VHSV-IVb positive. These involve VHS-susceptible species (OIE 2009) including sardines, *Sardina pilchardus*, from the North Atlantic and Mediterranean Sea, Pacific herring, *Clupea pallasi*, from the Pacific Northwest, and both rainbow smelt, *Osmerus mordax*, and lake herring, *Coregonus artedi*, from the Great Lakes (Arkush et al. 2006, Herve-Claude et al. 2008).

Despite the risks of virus introduction, frozen baitfish are not regulated as stringently as their live counterparts in the United States. For example, the USDA-APHIS Interim Rule (2008) was established to prevent the further distribution of VHSV-IVb; however, these regulations do not pertain to frozen baitfish. In response to these threats, Minnesota and Wisconsin have recently established new regulations specifically for the use of frozen baitfish in state waters (Minnesota Rules: Part 6262.0577, subpart 2; Wisconsin Statutes: Section 10, NR 20.08), herein referred to as the current regulatory options. All frozen baitfish originating from VHSV-positive waters or those with an unknown health history must be processed to inactivate infectious virus prior to use. The approved treatment methods include, submersion in either isopropyl alcohol or mineral oil for 14 days, being covered in a salt and borax mixture for 14 days, or dehydration followed by heating to 60°C. Isopropyl alcohol is a well-known broad spectrum antimicrobial, effective against enveloped viruses like VHSV (Danner & Merrill 2006, Perry & Caveney 2012); however, it is primarily used as a surface disinfectant. The

ability of isopropyl alcohol to sufficiently saturate the whole fish is unknown. Mineral oil is used to interfere with the transmission and retention of plant viruses (Wang & Pirone 1996); however, the effectiveness of mineral oil in animals or against VHSV is unknown. Furthermore, it seems unlikely that the highly hydrophobic mineral oil would penetrate the fish. The salt and borax treatment preserves the fish by drawing the water from the tissue, consequently dehydrating and inactivating the virus. The treated fish can then be rehydrated to regain their size (Thorarinsdottir et al. 2011). Similarly, dehydrating the whole fish removes the water, with any remaining infectious virus inactivated by heating to 60°C.

In this study we evaluate treatment techniques designed to inactivate VHSV in previously frozen baitfish. Our study includes both novel and standard industry methods.

4.2 Methods

4.2.1 Experimental infection. Bluegills, *Lepomis macrochirus*, were obtained from a commercial fish farm with a negative history of VHSV by annual farm-level inspection (USFWS and AFS-FHS 2010). The average length and weight (sd) were 11.96 cm (0.80 cm) and 27.10 g (1.12 g), respectively. The fish (n = 280) were transported live to the University of Arkansas at Pine Bluff, Fish Disease Research Laboratory and held in a biosecure facility. The fish were divided into two 300-L recirculating systems and acclimated for 5d at approximately 10°C.

The virus used in the experimental exposure was VHSV-IVb (Lumsden et al. 2007) propagated in fathead minnow cells. Prior to challenge, all fish were anesthetized

with MS-222 (tricaine methanesulfonate; Western Chemical, Inc., Ferndale, Washington). Each fish then received an intraperitoneal (IP) injection of 100µl of VHSV-IVb stock diluted 1:10 in Hank's Balanced Salt Solution (HBSS; Sigma, St. Louis, Missouri) producing a final infective dose of approximately 1 x 10^4 $TCID_{50}$ per fish. The fish were monitored daily for 7 d for clinical signs of VHS. This time period was selected based on data from similar challenge experiments (Goodwin & Merry 2011). Moribund or dead fish were removed from the aquaria daily and frozen at -80°C. Seven days post-injection, surviving fish were euthanized with an overdose of MS-222 and then frozen at -80°C. All fish were sent overnight on dry ice to the Minnesota Veterinary Diagnostic Laboratory (MVDL) to be treated and subsequently tested for the presence of infectious VHSV by virus isolation. Another 40 fish that were not challenged with VHSV-IVb from a known VHSV-negative waterbody in Minnesota were shipped to the MVDL on frozen ice pack and then frozen at -80°C. The latter served as negative control fish.

4.2.2 VHSV disinfection treatments. Upon receipt at the MVDL, all infected bluegills were thawed to room temperature (22°C), mixed on a lab bench, and randomly allocated to seven groups of 40 fish each. The fish underwent six different treatment regimens, based on current regulatory requirements for the treatment of frozen baitfish and standard industry practices. One group of challenged fish served as the positive control and was left untreated. The additional group of bluegills not exposed to VHSV-

IVb served as the negative control. Unless otherwise stated, all treatment groups were held at 22°C in a ventilated hood for the duration of the treatment.

Positive Control – 40 bluegills challenged with VHSV-IVb underwent no additional treatment and were immediately tested. To examine potential differences between the viral load of dead/moribund fish (samples 1-10) and euthanized fish (samples 11-20) post-challenge, like fish were pooled (Table 1).

Negative control – 40 bluegills not challenged with VHSV-IVb underwent no treatment and were immediately tested.

Freeze/thaw – 40 bluegills underwent three cycles of freeze – thaw. These cycles were completed in a standard freezer at -20°C for 16h and slow thawed at 22°C for 8h, over a span of three days. Fish were pooled in one freezer bag during treatment. The duration and temperature of the treatments were to sufficient to thoroughly freeze and thaw all fish. These are the standard industry methods frozen baitfish would be subjected to prior to use by the consumer in Minnesota and Wisconsin (Minnesota Department of Natural Resources (MNDNR), personal communication).

Isopropyl alcohol – 40 bluegills were submerged in 70% isopropyl alcohol for 14 days in a sealable container. The ratio was approximately 542g of fish to 1.5 L of isopropyl alcohol (current regulatory option).

Mineral oil – 40 bluegills were submerged in mineral oil (Vi-Jon; Smyrna, Tennessee) for 14 days in a sealable container. The ratio was approximately 542g of fish to 1.5 L of mineral oil (current regulatory option).

Salt and borax – 40 bluegills were thoroughly coated in a salt and borax mixture and let stand for 14 days. The mixture consisted of 900g of non-iodized salt and 100g of borax (current regulatory option).

Dehydration – 40 bluegills were dehydrated in a dehydrator (Oster, Model FPSTDH0101, Sunbeam Products, Inc.; Boca Raton, Florida) at 50°C for eight hours. This amount of time was shown to be sufficient to completely dry the bluegill when visually examined during necropsy (novel method).

Dehydration and heat – 40 bluegills were dehydrated at 50°C for eight hours. Following dehydration, the fish were placed in a laboratory oven at 60°C for 15 min (current regulatory option).

4.2.3 Virus isolation. Upon completion of each treatment, the fish were rinsed with tap water to remove treatment residue. Some fish treated in mineral oil could not be rinsed because an advanced state of decomposition rendered them too fragile. Kidney and spleen tissue was removed from groups of two fish each and pooled for a total of 20 pools per treatment group. Given the small size of each fish, pooling was necessary to acquire sufficient sample size for virus isolation. Necropsy tools were disinfected for 2 min in a 1:256 dilution of broad spectrum antimicrobial (Synergize, Preserve International; Reno, Nevada), then rinsed with tap water and dried between each pool to prevent contamination.

Sample preparation and virus isolation protocols followed the methods described by the USFWS and AFS-FHS Blue Book (2010). Briefly, a 10% suspension of the tissue

pool was prepared in 2 mL of HBSS (Sigma, St. Louis, Missouri). The suspension was homogenized in a stomacher for 30 sec, then centrifuged at 4°C for 15min at 2,900 x g. An equal amount of antibiotic medium was added to the supernatant and thoroughly mixed. The suspension was incubated for 2 hours at 15°C and re-centrifuged for 15min at 2,900 x g. The resulting supernatant was used for virus isolation.

Monolayers of epithelioma papulosum cyprini (EPC) cells (Fijan et al. 1983, Winton et al. 2010) were prepared in 24-well microtiter plates. When monolayers were 80% confluent, the cell culture medium was replaced with 100 µl/well of the sample suspension. The cultures were then incubated at 15°C for 60 min to allow for viral adsorption. Maintenance medium was added and the plates were incubated at 15°C for 14 days and monitored three times per week for the appearance of cytopathic effect (CPE). Unless CPE was observed, all samples were blind passaged at day 14. If no CPE appeared after an additional 14 d second passage, the sample was determined to be negative and no additional testing was performed. If CPE was observed, the original material was tested by real-time reverse transcriptase PCR (rRT-PCR) for confirmation (Phelps et al. 2012). A VHSV-IVb isolate (Great Lakes reference strain: MI03) was used as a positive control to monitor the sensitivity of the cell line to the virus.

4.3 Results

4.3.1 Experimental infection. During the 7 d VHSV-IVb challenge at 10°C, 150 bluegills were removed due to mortality or morbidity. The surviving 130 bluegills were euthanized on day 7. Although slightly more apparent in moribund and dead fish, clinical

signs of VHS (petechial and ecchymotic hemorrhage of the skin, vent, base of fins, and eye) were observed in most of the challenged fish (Figure 4.1).

4.3.2 VHSV disinfection treatments. The overall condition of the fish post-treatment varied between groups based on gross exam (Figure 4.1). The isopropyl alcohol treatment preserved the fish well and appeared to penetrate the whole fish. The mineral oil treatment was not effective in preserving the fish and resulted in severe decomposition and odor. The salt and borax, and dehydration treatments effectively preserved the fish by removing water from the tissue. The freeze/thaw treatment produced the most natural product.

4.3.3 Virus isolation. VHSV-IVb was isolated from all positive control fish on the first cell culture passage. No virus was isolated from the negative control fish. Infectious VHSV-IVb virus was isolated from 14/20 (70%) samples from the freeze/thaw treatment. Of the remaining treatment groups, all disinfection methods were successful in inactivating VHSV-IVb from frozen bluegills (Table 4.1). There was no cytotoxic impact on the virus isolation (premature cell death or ambiguous CPE) as a result of any treatment residue. All tissue samples that produced CPE in culture were also positive by rRT-PCR. The mean (sd) Ct for the positive control group was 22.54 (2.63) and the freeze/thaw treatment was 27.14 (2.45).

4.4 Discussion

The current industry standard of freezing baitfish is not sufficient to inactivate infectious VHS-IVb. These methods did however reduce the positive samples by 30%. While the purpose of freezing the baitfish is not necessarily to inactivate pathogens, it has been suggested it may be sufficient for baitfish populations with low viral loads or effective in appropriate environmental conditions (Arkush et al. 2006). However, for high-risk pathogens, like VHSV-IVb, additional precautions are warranted in areas where transmission is likely. This is particularly important given that frozen baitfish are often harvested from waters of unknown disease history, or in some cases known-VHSV-positive waters, and fed to naïve populations. However, given the diversity of baitfish and their use, a thorough risk assessment should be performed for each scenario to justify additional processing requirements.

The four methods (isopropyl alcohol, mineral oil, salt and borax, and dehydration and heat) currently approved by the Minnesota and Wisconsin Department of Natural Resources were shown to be effective in reducing infectious VHSV-IVb beyond the limit of detection by virus isolation. Confidence in these methods is further increased due to the high viral loads of the challenged fish. Presumably, frozen baitfish harvested from the wild would carry considerably less virus under normal conditions. It is also important to note that the dehydration treatment without subsequent heating at 60°C was also shown to be effective.

In locations where these treatments are currently required, there has been some pushback from the frozen bait industry and anglers (MNDNR, personal communication). They understand the importance of these regulations and the risk VHSV-IVb poses to

wild and farmed fish populations, but adapting to new rules is challenging. In addition to increased processing costs, these treatments are time consuming and require additional handling. This burden would increase with the salt and borax or dehydration treatments because fish would need to be rehydrated to eliminate buoyancy issues. In addition, the effect of the treatment on the quality and palatability of the bait is potentially problematic and further evaluation is needed. For example, the mineral oil treatment led to severe decomposition of tissue and an extreme odor, resulting in a product likely not suitable for use as bait. We also have concerns about the potential for clostridial growth and botulism toxin production in these fish decomposing under anaerobic conditions.

Dehydration followed by rehydration is rapid and effective, and therefore, seems to be the best option for inactivating VHSV-IVb from frozen baitfish while maintaining their usefulness as bait. However, this method does pose a risk if the fish is not sufficiently dehydrated. Further species-specific investigation is needed to identify appropriate dehydration times and necessary temperatures for traditional frozen baitfish, like Pacific herring and sardines. In addition, technological advancements or anti-viral products that do not affect baitfish taste or body condition should be considered for development.

4.5 References

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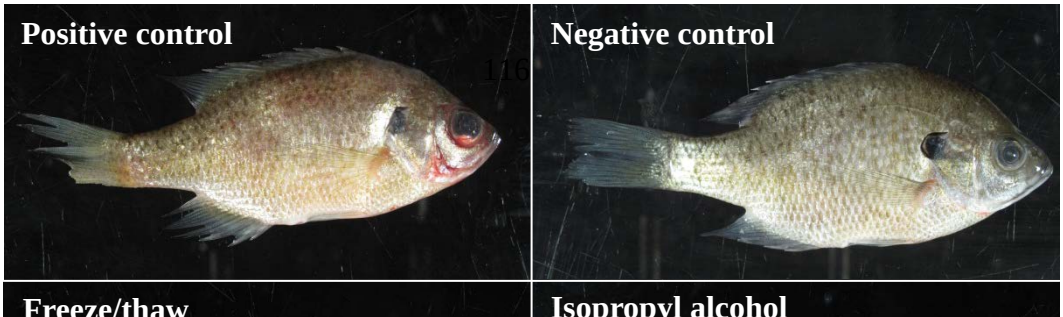
Table 4.1. Virus isolation and rRT-PCR results of various viral hemorrhagic septicemia virus (VHSV) disinfection treatments on previously frozen VHSV-IVb-positive bluegills. Each sample (1-20) contains a pool of two bluegills (n = 40) for each treatment. Numeric values indicate the rRT-PCR threshold cycle (Ct) of cell-culture-positive samples. Minus signs show samples that were negative by cell culture (these were not tested by rRT-PCR). The average Ct (sd) of the positive control and freeze/thaw treatments were 22.54 (2.63) and 27.14 (2.45), respectively.

Sample Number	Positive Control	Negative Control	Freeze/ Thaw	Isopropyl Alcohol	Mineral Oil	Salt + Borax	Dehydration	Dehydration + Heat
1	21.30	-	-	-	-	-	-	-
2	21.20	-	-	-	-	-	-	-
3	20.71	-	-	-	-	-	-	-
4	21.56	-	25.23	-	-	-	-	-
5	21.82	-	-	-	-	-	-	-
6	20.83	-	28.98	-	-	-	-	-
7	22.80	-	25.50	-	-	-	-	-
8	22.16	-	24.75	-	-	-	-	-
9	20.77	-	25.10	-	-	-	-	-
10	28.08	-	27.40	-	-	-	-	-
11	20.35	-	24.65	-	-	-	-	-
12	20.68	-	31.08	-	-	-	-	-
13	28.51	-	24.69	-	-	-	-	-
14	21.39	-	28.79	-	-	-	-	-
15	22.34	-	26.80	-	-	-	-	-
16	21.23	-	-	-	-	-	-	-
17	26.17	-	30.34	-	-	-	-	-
18	22.16	-	25.60	-	-	-	-	-
19	27.12	-	31.06	-	-	-	-	-
20	20.33	-	-	-	-	-	-	-

Figure 4.1. External condition of various viral hemorrhagic septicemia virus (VHSV) disinfection treatments on previously frozen VHSV-IVb – positive bluegills. Each image is a representative fish from the various treatment groups. Treatments included: positive control, negative control, freeze/thaw, isopropyl alcohol, mineral oil, salt and borax pre rinse, salt and borax post rinse, and dehydration with and without subsequent heat.



SECTION D: IMPROVED MANAGEMENT

CHAPTER 5

RISK-BASED MANAGEMENT OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV-IVb) IN MINNESOTA

Viral hemorrhagic septicemia virus (VHSV-IVb) is a serious disease of fish in the Great Lakes region of North America. In addition to the numerous large scale mortality events of wild fish, the emergence of VHSV has resulted in an unprecedented regulatory response to protect both farm raised and wild fish populations. However, monitoring or regulating all risk factors for the continued transmission of VHSV is a difficult task. A semi-quantitative risk assessment model was utilized to focus VHSV management efforts

in Minnesota. The risk of VHSV introduction to major watersheds in Minnesota was directly correlated to the proximity to Lake Superior, the only VHSV-positive waterbody in the state. Although the current regulations are uniform across Minnesota, the risk varied for specific locations within the watersheds. For example, the introduction of game fish for stock enhancement (a common fisheries management practice) is a significant factor to determine the risk of VHSV introduction into public waterbodies and waterbodies frequently used for wild baitfish harvest. Aquaculture facilities with strict biosecurity programs and frequent health inspections received the lowest risk scores and were largely considered protected or low risk for VHSV introduction. These results suggest the current management strategy, based on political boundaries, should be re-evaluated. A risk-based management strategy would better allocate efforts to watersheds or specific waterbodies at higher risk and relax efforts in areas of lower risk of VHSV introduction in Minnesota.

5.1 Introduction

Viral hemorrhagic septicemia (VHS) is a highly contagious viral disease causing high morbidity and mortality in a wide range of marine, brackish, and fresh water fish species (Kim and Faisal 2011). The disease is caused by viral hemorrhagic septicemia virus (VHSV), which belongs to the genus Novirhabdoviris within the Rhabdoviridae family. Due to the significant impact on farmed and wild fish populations, VHSV is

listed by the World Organization for Animal Health as a reportable pathogen (OIE 2012). In 2005, a new strain of VHSV (VHSV-IVb) was identified in the Great Lakes (Elsayed et al. 2006, Lumsden et al. 2007). This novel virus spread rapidly through the region, resulting in numerous mass mortality events in wild fish throughout the Eastern Great Lakes and inland lakes of Michigan, New York, and Wisconsin (Kim and Faisal 2011, Thompson et al. 2011). Although Lake Superior has not experienced a known large mortality event, the virus has indeed been isolated from resident fish (Faisal et al. 2012).

The rapid spread of VHSV-IVb in the Great Lakes region has heightened concerns for the transmission of this virus to naïve populations. These concerns are elevated due to a number of factors, including the geographic range of VHSV, the interconnectedness of the Great Lakes watersheds, routine movement of fish, and the presence of potentially asymptomatic carriers. Many of the possible transmission routes are not fully understood and hence cannot be effectively managed. However, knowledge of transmission routes is expanding (VHSV Expert Panel 2010). Regardless, monitoring or regulating all risk factors for the transmission of VHSV is an infeasible task. Interventions, such as movement restrictions and increased diagnostic testing, are costly, time consuming, and can easily prohibit business activity and recreational enjoyment. Therefore, a targeted risk-based management strategy is an ideal solution for the most feasible and cost-effective approach to manage VHSV (Gustafson et al. 2010. Thrush et al. 2011).

Implementation of a risk assessment model can have wide reaching outcomes for aquatic animal health (Stephen 2001, Stark et al. 2006, Peeler and Taylor 2011, Thrush et

al. 2011). For VHSV, the allocation of limited funds can be directed to watersheds or individual waterbodies identified as 'high-risk' for introduction. Strategies may include additional surveillance testing, regulatory action, increased educational effort, or other intervening management strategies. In addition, the identification of risk, along with active surveillance, can identify areas free of disease where regulatory burden could potentially be relaxed, or at a minimum, re-evaluated (Gustafson et al. 2010). For example, an aquaculture facility with several years of VHSV-negative tests and a strict biosecurity program could be subjected to lower regulatory standards (i.e. less frequent inspections) than baitfish harvested from the wild where the disease status is unknown.

While VHSV-IVb was first isolated from Lake Superior in 2009, no inland waters of Minnesota have been identified as VHSV-positive, despite five years of active surveillance and regulatory testing (Faisal et al. 2012, Phelps et al. 2012). However, Minnesota remains one of the most burdensome states to the aquaculture industry in regards to VHSV, requiring costly and time consuming annual inter and intra state inspections of all susceptible species prior to movement (USDA 2008, Minnesota Statutes 2012, Section 17.4991, Subdivision 3). These regulations, combined with increasingly limited funds to manage the disease have created the need for current policies to be re-examined. Risk-based management would help to identify high-risk populations where interventions should be focused.

Identifying VHSV risk factors is a time consuming task in itself, involving decades of research and expert opinion. The present case study utilized the risk factors identified by the VHSV Expert Panel and Working Group (2010). Their assessment

identified and quantified nine of the most likely VHSV exposure routes and their associated likelihood ratios. While by no means a complete list, the factors were considered the highest ranked actual or perceived risks to facilitate the spread of VHSV in the Great Lakes region. This case study examines the risk of VHSV introduction for watersheds in Minnesota, as well as specific wild and farmed populations, to inform the State's long-term management strategy. This approach will also serve as a model for a broader VHSV management strategy throughout the Great Lakes region.

5.2 Materials and methods

5.2.1 Identifying locations. The exposure risk of VHSV was evaluated for two types of locations. The first location type examined was watersheds within the State of Minnesota. There are 12 four-digit hydrologic unit code (HUC-4) watersheds in Minnesota (0401, 0701, 0702, 0703, 0704, 0706, 0708, 0710, 0902, 0903, 1017, 1023) (USGS 2012). Since these areas are large and are comprised of diverse aquatic populations, the demographics for each watershed were generalized based on the dominant characteristics of wild fish populations in the area; however, proximity to a risk factor was based on the nearest location within the watershed. Demographics included factors associated with the potential for introduction of VHSV, such as fish introductions and geographic proximity to positive waterbodies. To visually demonstrate the risk of VHSV introduction to Minnesota watersheds, a GIS-based heat map was created using R (Version 2.15.1).

The second location type examined the site-specific risk of ten wild and farmed fish populations distributed across Minnesota. Data was collected from on-site inspection, manager survey, and the Minnesota waterbody database. Five wild fish populations identified as "high-priority" by the Minnesota Department of Natural Resources, based on recreational use and perceived VHSV risk, were evaluated including, Big Stone Lake (Big Stone County), Lake Minnetonka (Hennepin County), Lake Pepin (Goodhue County), Leech Lake (Cass County), and the St. Louis River (St. Louis County). Two waterbodies frequently used for wild baitfish harvest were evaluated, including Battle Lake (Otter Tail County) and Lake of the Woods (Lake of the Woods County). Three aquaculture facilities raising both VHSV-susceptible and non-susceptible species were also examined. The precise locations of the aquaculture facilities is confidential.

5.2.2 Identifying risk factors. The risk factors for the introduction of VHSV were based on those established by the VHSV Expert Panel and Working Group (2010). These factors were identified based on the experience of participants as experts in the field. Briefly, the expert panel was asked to individually identify the top 10 potential watershed-level risk factors that would likely be associated with a new introduction of VHSV-IVb in the United States. The resulting list of factors (n = 28) was scored (1-10) and narrowed down to the nine top risk factors. These were further evaluated to assign response categories and associated likelihood ratios (LR). For example, close proximity to a VHSV-positive waterbody was identified as a major risk factor. Thus, locations

further than 500 km were at lower risk (LR = 0.15) than waterbodies within 100 km (LR = 6.25). To reduce asymmetrical bias (score of 10 was the maximum), the median of the square root transformation of each LR was used for this study (Table 1).

5.2.3. Risk assessment model. For each location, the likelihood of VHSV introduction, also known as the risk score (RS), was determined. This was performed by evaluating each risk factor for a given location and selecting the associated LR. The product of the combined LR for a given location provides the RS for potential VHSV-IVb introduction. Values greater than one suggest the potential for VHSV introduction is likely, while values less than one suggests the location is protected. Likelihood ratios or RS equal to one indicate the introduction risk is neutral (VHSV Working Group 2010). For purposes of this case study, we have assigned RS values greater than 5 to be "high risk", 2-5 to be "moderate risk", 0.8 to 2 to be "low risk", 0.3 – 0.8 to be "minimal risk", and values less than 0.3 to be "protected".

A map was generated using R to overlay the watershed introduction risk with the locations of previous surveillance testing. The previous surveillance sites included (Phelps et al. 2012) represent the majority of private aquaculture production and wild baitfish harvest in Minnesota.

5.3 Results

Risk scores for all 12 HUC-4 watersheds in Minnesota were calculated, with scores ranging from 0.30 to 18.96 (Figure 1). All risk factors were constant across all

watersheds, except for linear distance and hydrologic connectivity to a VHSV-positive waterbody. An overlay of the watershed risk scores with the VHSV survey sites from 2010-2011 shows the majority of aquaculture facilities and wild baitfish harvest are in watersheds with minimal risk (RS = 0.77) of VHSV introduction, while most of the remaining locations are in watersheds with low risk (RS = 1.92) (Figure 2).

The RS for specific sites varied from their associated watershed risk score (Table 2). In addition to linear distance and hydrologic connectivity to a VHSV-positive waterbody, transfer of fish for stocking/culture, controlled fomite exposure, and the use of bait influenced the risk of VHSV introduction. The RS for five public waterbodies ranged from 0.30 to 16.56. The RS for the two waterbodies frequently used for wild baitfish harvest were 0.30 and 0.77. The three private aquaculture production facilities were the most protected of the sites evaluated, with RS ranging from 0.00 to 0.17.

5.4 Discussion

In Minnesota, the current management strategy considers VHSV introduction risk equal across the state, regardless of unique location demographics, control measures, or disease-free history. In this case study, we estimated the risk level for introduction of VHSV in Minnesota watersheds and specific locations. The RS variation between watersheds was strongly associated with the proximity to Lake Superior, the only VHSV positive waterbody in Minnesota. Outside of the Lake Superior watershed, much of the state is considered ‘protected’ to ‘low risk’ for VHSV introduction. While these results

may be intuitive, current management strategies at the State and Federal levels are based on political boundaries, not on established hydrologic units.

All three aquaculture facilities evaluated had a lower risk score than their associated watershed and were considered protected. This was achieved by relatively simple procedures, such as preventing fomite exposure from waterbodies with an unknown health status and limiting the introduction of fish into the facility. As an example of where regulations could be relaxed, one farm located on the Minnesota – South Dakota border was included in this case study because this area provides a significant number of bait and sportfish to Minnesota and beyond. Despite the distance from Lake Superior (>500 km), containment and other biosecurity measures, this farm is currently subjected to the same regulations in Minnesota as wild baitfish harvested within the Lake Superior watershed. While on-farm vigilance should remain high to prevent even the unlikely introduction of VHSV, compromises could be reached to maintain regulatory confidence and promote production activities. For examples, as an aquaculture facilities demonstrate repeated years of VHSV-free status, voluntarily implements strict biosecurity programs, and managers consider VHSV introduction risk, a graduated testing regime (i.e. fewer fish or less frequent intervals) could be implemented.

One important aspect of risk-based management is inclusion of all stakeholder opinions and values to establish acceptable levels of risk. While these considerations were not accounted for as part of this case study, we can speculate based on current management strategies in Minnesota. Introducing fish for stock enhancement has been

an essential aspect of fisheries management and helps to support a multi-billion dollar recreational fishing industry in Minnesota. However, this activity is a risk factor for VHSV introduction. This was demonstrated by the lower RS for specific public waterbodies that were not stocked, compared to their associated watershed. While provisions have been implemented to lower the risk (i.e. routine VHSV testing of stocked fish), the management demand for stocking public waters outweighs the risk of VHSV introduction in Minnesota. Likewise, the demand for baitfish, much of which is wild caught from public waters, overrides the perceived or actual risk of VHSV introduction. The threshold for tolerable risk is often a moving target with a diversity of stakeholder values; however, the implementation of a risk-based management approach should scientifically justify the eventual decision.

Based on this case study for Minnesota, we feel a risk-based management approach should be evaluated for other states throughout the Great Lakes region. Focusing the limited available resources to manage VHSV to high risk areas is essential. We believe we are at a point where current management plans should be reevaluated and scientifically justified, to effectively and efficiently prevent the spread of VHSV.

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Table 5.1. Viral hemorrhagic septicemia virus (VHSV-IVb) introduction risk factors and associated likelihood ratios, as determined by the VHSV Expert Panel (2010). To reduce asymmetrical bias, the likelihood ratio is the median of the square root transformation. Likelihood ratios greater than one suggest the response category is a likely risk for VHSV introduction, equal to one suggests the risk is neutral, and values less than one suggest the response category is protected.

Risk Factor Response Category	Likelihood Ratio
Hydrologic connectivity to VHSV-positive waterbody	
Connected, with fish movement	3.16
Downstream, no fish movement	1.41

Upstream, no fish movement	0.71
No connection	0.32
Linear distance to VHSV-positive waterbody	
< 100 km	2.50
100 - 500 km	1.00
> 500 km	0.39
Known-susceptible species	
Yes, with known congregation areas	2.00
Yes, but no known congregation areas	1.22
No	0.24
Conducive water temperatures	
Yes, cool to cold water	1.50
No	0.47
Fomite exposure	
Yes, shared traffic or wastes	2.24
Yes, but limited by education or regulation	1.00
No	0.39
Live fish transfer, bait	
Yes, without testing	2.65
Yes, with testing	1.00
No, transfers prevented	0.34
Live fish transfer, culture/stock	
Yes, without testing	2.45
Yes, with testing	1.00
No, tranfers prevented	0.39
Frozen fish transfer	
Yes, without testing	2.45
Yes, with testing	1.00
No, tranfers prevented	0.58
Regulatory framework	
Sufficient	0.80
Insufficient	1.34

Table 5.2. Viral hemorrhagic septicemia virus introduction risk scores for specific locations, including five public waterbodies, two waterbodies frequently used for wild baitfish harvest, and three aquaculture farms.

Site	**St. Louis River**	**Leech Lake**	**Farm**	**Big Stone**	**Farm**	**Lake Pepin**	**Lake Minnetonka**	**Battle Lake**	**Lake of the Woods**	**Farm**
County	St. Louis	Cass	Aitkin	Big Stone	Douglas	Goodhue	Hennipen	Otter Tail	Lake of the Woods	Brookings, SD
HUC-4	0401	0701	0701	0702	0702	0704	0704	0902	0903	1017
Classification	Public	Public	Culture	Public	Culture	Public	Public	Bait harvest	Bait harvest	Culture
Watershed Risk Score	**18.96**	**1.92**	**1.92**	**0.77**	**0.77**	**0.77**	**1.92**	**0.77**	**1.92**	**0.30**
Site Risk Score	**16.56**	**0.77**	**0.00**	**0.77**	**0.17**	**0.30**	**0.77**	**0.77**	**0.30**	**0.03**
Risk Factors included in Risk Score										
Hydrologic connectivity	3.16	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32
Linear distance	2.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.39
Known-susceptible species	2.00	2.00	0.24	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Conducive water temperatures	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Fomite exposure	2.24	1.00	0.39	1.00	0.39	1.00	1.00	1.00	1.00	0.39
Live fish transfer, bait	1.00	1.00	0.34	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Live fish transfer, culture/stock	0.39	1.00	0.39	1.00	1.00	0.39	1.00	1.00	0.39	0.39
Frozen fish transfer	1.00	1.00	0.58	1.00	0.58	1.00	1.00	1.00	1.00	0.58
Regulatory framework	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80

Figure 5.1. Risk of Viral hemorrhagic septicemia virus introduction to Minnesota watersheds. Numbers identify watersheds based on the level 4 hydrologic unit code.

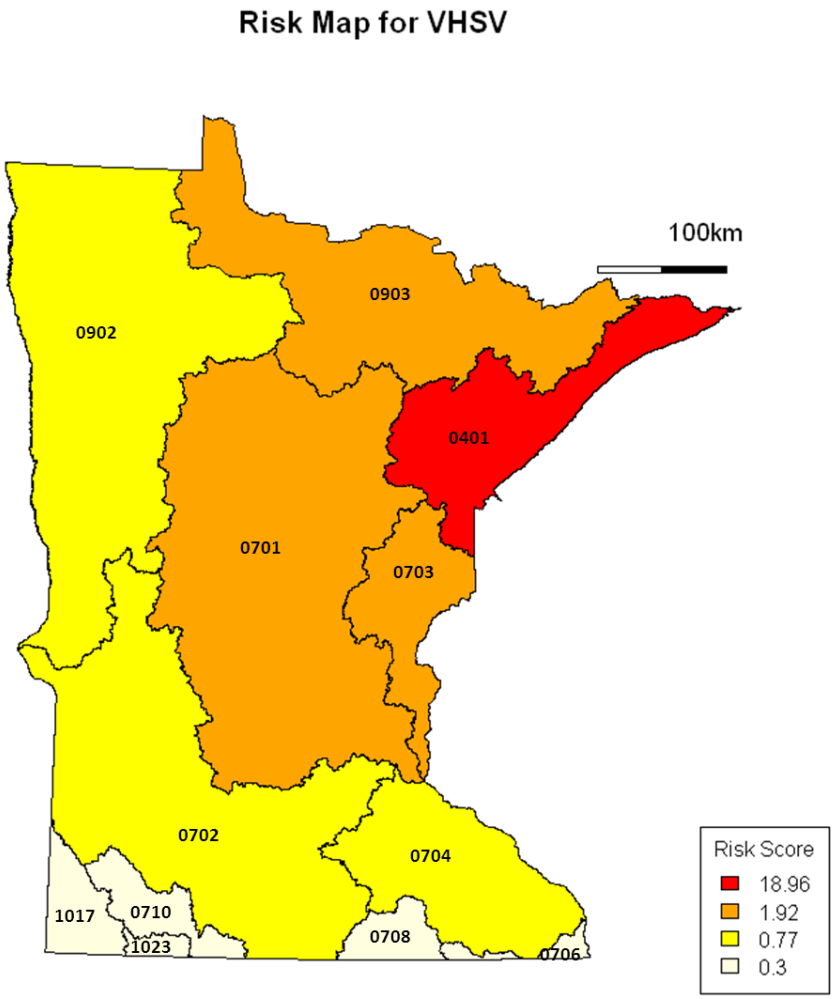
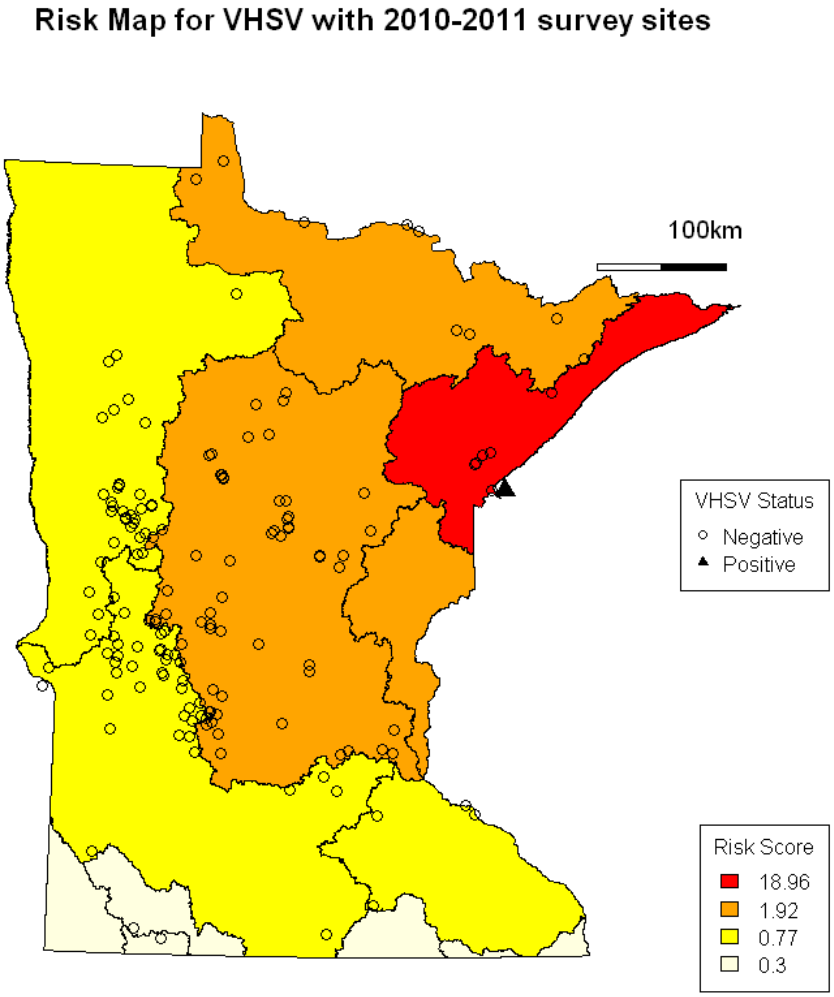


Figure 5.2. Risk of viral hemorrhagic septicemia virus introduction to Minnesota watersheds with locations of aquaculture and wild baitfish activity. Open circles indicate a VHSV-negative health inspection within the last two years. The filled triangle on Lake Superior indicates the only VHSV-positive waterbody in Minnesota.



SECTION E: GENERAL CONCLUSIONS

Viral hemorrhagic septicemia virus (VHSV) is a highly lethal and significant threat to farm raised and wild fish populations. The elusive nature and high adaptability of this virus have allowed for its worldwide dispersal to freshwater and marine environments. The recent emergence of a new strain, VHSV-IVb, in the Great Lakes region has resulted in numerous mass mortality events and the loss of millions of fish. Most of the known VHSV-IVb susceptible species such as walleye, muskellunge, yellow perch, trout, and baitfish, are both ecologically and economically important to the region and thus preparing Minnesota to respond to this disease is critical. To that end, the focus of this dissertation was to improve diagnostic and management strategies for VHSV-IVb. This has better prepared Minnesota to rapidly respond to a VHSV outbreak, to prevent the introduction of the virus into farm raised and wild fish populations, and to scientifically justify future policy.

E.1 General Conclusions

E.1.1 Chapter 1. To rapidly respond to an outbreak and improve sensitivity over traditional assays, a modified one-step real-time reverse transcriptase PCR (rRT-PCR) for the detection of VHSV was developed in Chapter 1. The assay was shown to be twice as sensitive as the gold standard, cell culture. Since VHSV can persist in wild fish populations at low levels without producing clinical signs of disease, this improved sensitivity is critically important to prevent the spread of VHSV. In addition, rRT-PCR significantly decreased the turnaround time from 30 days for cell culture to 2 days.

This assay was used to better understand the current distribution of VHSV in Minnesota waters. During a two-year survey, more than 115 locations, representing approximately 7,500 fish, were tested by cell culture and rRT-PCR. This broad survey found all locations to be VHSV-negative, thus we can confidently predict inland waters of Minnesota remain free of the disease. Applications for these data have been wide-reaching including easing public concern for this emerging fish disease, improving selection criteria for future testing, and informing management recommendations. This survey also satisfied the regulatory requirements for the intra- and interstate movement of farm raised fish in Minnesota during the survey period. Consequently, industry support for Chapter 1 was very strong in Minnesota.

E.1.2 Chapter 2. The development and use of rRT-PCR for widespread surveillance has increased in recent years; however, assay validation has rarely considered the type of sample available for testing. Potential sample-induced PCR inhibition was evaluated in Chapter 2. Using the rRT-PCR assay by Jonstrup et al. (2012), various tissue types, including kidney/spleen, entire viscera, and ovarian fluid did not affect the detection of VHSV-IVb. The rRT-PCR by Phelps et al. (2012) resulted in inconsistent results, attributed to poor assay robustness across multiple diagnostic laboratories. While Chapter 2 provided evidence that the use of rRT-PCR was a suitable method for widespread surveillance studies, it highlighted the importance of thorough validation.

E.1.3 Chapter 3. Chapter 3 of this dissertation developed a Fish Health Collector Workshop and associated Manual. The Workshop certified a sufficient number of fish health collectors to perform regulatory inspections related to VHSV – previously a significant bottleneck to the movement of fish in Minnesota. In addition, these certified fish health collectors completed the necessary field work for the success of the VHSV survey performed in Chapter 1.

E.1.4 Chapter 4. Chapter 4 demonstrated the current industry standard of freezing baitfish for preservation is not sufficient to inactivate infectious VHS-IVb. The four methods (isopropyl alcohol, mineral oil, salt and borax, and dehydration and heat) currently approved by the Minnesota and Wisconsin Department of Natural Resources were shown to be effective in reducing infectious VHSV-IVb beyond the limit of detection by cell culture; however, not all produce a suitable product (mineral oil treatment) for use as bait post-treatment. Dehydration followed by rehydration is rapid and effective, and therefore, seems to be the best option for inactivating VHSV-IVb from frozen baitfish while maintaining their usefulness as bait.

E.1.5 Chapter 5. In Minnesota, the current management strategy considers VHSV introduction risk equal across the state, regardless of unique location demographics, control measures, or disease-free history. Chapter 5 demonstrated a strong association between introduction risk and the proximity to Lake Superior, the only VHSV-positive waterbody in Minnesota. Outside of the Lake Superior watershed, much

of the state is considered ‘protected’ to ‘low risk’ for VHSV introduction based on this risk analysis, including much of the aquaculture industry. Furthermore, site specific intervention strategies (i.e. farm biosecurity) effectively lowered the risk of VHSV introduction compared to their associated watershed. While these results may be intuitive, current management strategies at the State and Federal level are based on political boundaries, not established hydrologic units or on-farm control measures.

E.2 Recommendations

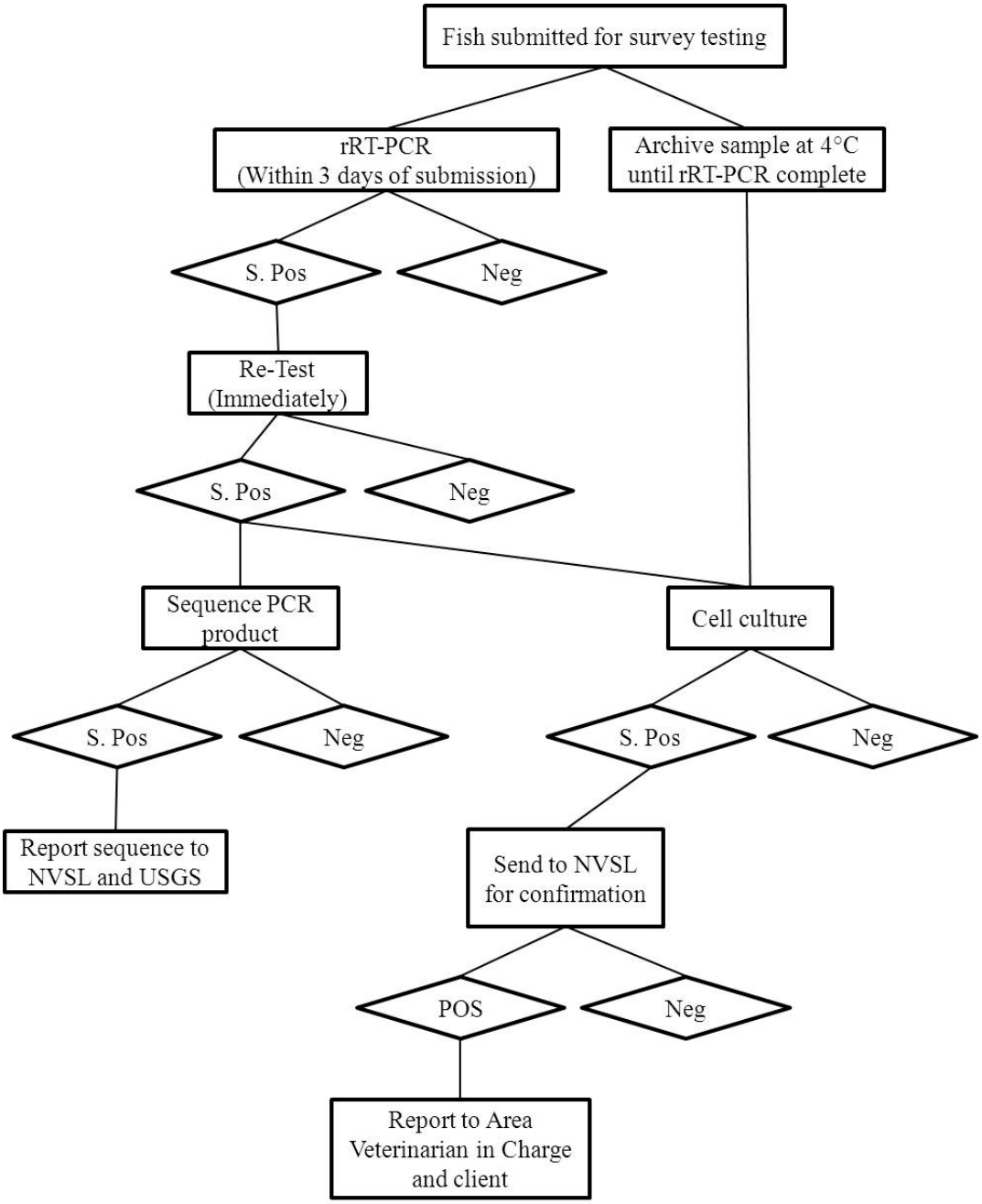
E.2.1 The use of rRT-PCR for the surveillance of VHSV. Despite the widespread use and acceptance of rRT-PCR for the detection and surveillance of many terrestrial animal diseases, the same cannot be said for diseases of aquatic animals. However, this dissertation has demonstrated the value and suitability of rRT-PCR as a diagnostic tool for the surveillance of VHSV. Rapid detection and high sensitivity are paramount to identify and effectively control a disease outbreak, regardless of the animal’s environment. This has been well demonstrated with the emergence of VHSV-IVb in the Great Lakes where asymptomatic carrier fish and/or contaminated fomites, water or other unknown vector has repeatedly moved the virus undetected to inland waterbodies. As technology continues to improve and appropriate evaluations are performed, rRT-PCR will inevitably become the assay of choice for the detection of VHS and other emerging diseases of aquatic animals.

Prior to widespread acceptance of rRT-PCR for the surveillance of VHSV, consideration must be made to the appropriate interpretation of results. Each possible

diagnostic scenario must be evaluated and reporting guidelines established. Premature reporting of results or lack of approved confirmation could lead to devastating outcomes. To date, no government agency (i.e. USDA, USFWS) or association (i.e. USAHA, AFS-FHS) has offered a recommendation for the interpretation of rRT-PCR results for the surveillance of VHSV in the United States. However, these risks

The following diagnostic flow chart was developed for Chapter 1 of this dissertation and is one option for the interpretation of rRT-PCR during VHSV surveillance (Figure E1). Positive rRT-PCR results should remain suspect until confirmed by virus isolation – the current gold standard. It is important that rRT-PCR be performed within 3d of sample submission to ensure the tissue homogenate remains suitable for cell culture in the event confirmation is needed. If virus isolation does not support the suspect-positive result, it should be considered "a population of interest, in need of further testing" or simply "negative" as it would have been without the use of rRT-PCR. A suspect-positive by rRT-PCR should not result in immediate depopulation or other regulatory action. In the event of conflicting results, other factors should be considered to warrant additional investigation, such as clinical signs of disease, previous testing history, disease risk factors, or the number of suspect-positive results. Additional action may include, re-testing the original material, re-sampling the population, sequencing the rRT-PCR product, and other confirmatory tests.

Figure E1. Diagnostic workflow and reporting plan for VHSV surveillance testing by rRT-PCR in Minnesota. Positive result not reported to regulatory authority until VHSV has been confirmed in cell culture by the National Veterinary Service Laboratory. S. pos = Suspect positive, Neg = Negative, POS = Positive.

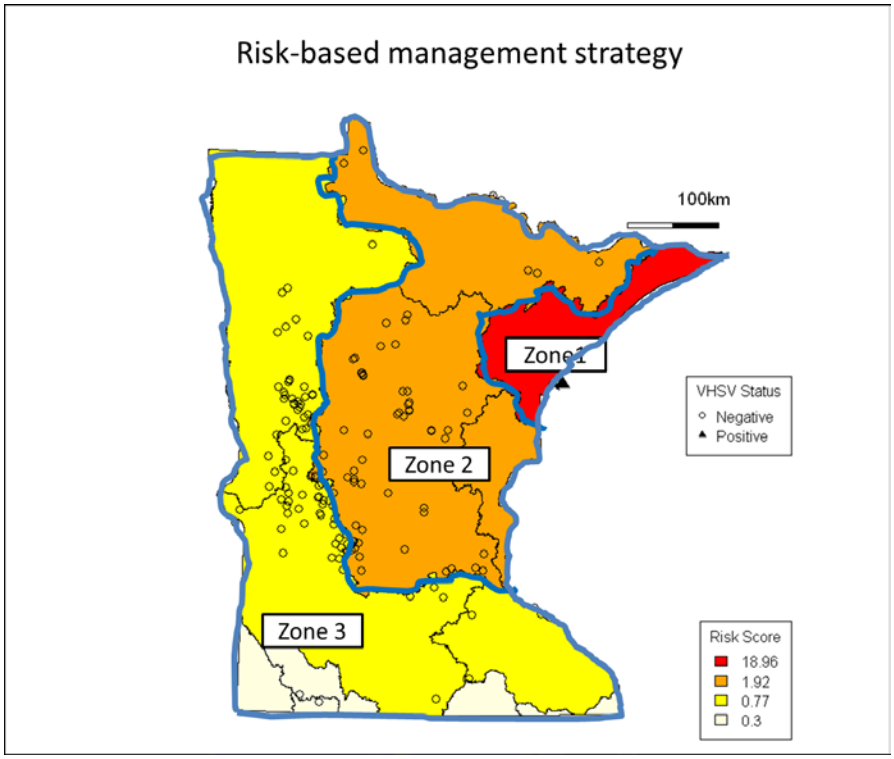


E.2.2 Treatment of frozen baitfish. Despite the well documented risks of virus introduction, frozen baitfish are not regulated as stringently as their live counterparts in the United States. Despite significant industry pushback for additional regulations, high-risk pathogens, like VHSV-IVb, warrant additional precautions in areas where transmission risk is high. This is particularly important given that frozen baitfish are often harvested from waters of unknown disease history, or in some cases known-VHSV-positive waters, and introduced to naïve populations. Given the diversity of baitfish and their use, a thorough risk assessment should be performed for each scenario to evaluate additional processing and regulatory requirements.

E.2.3 Risk-based management approach. Focusing the limited available resources to manage VHSV to high risk areas is essential. Based on the risk analysis performed in Chapter 5, it is clear a risk-based management approach should be considered for Minnesota. This could be achieved by the development of "VHSV Management Zones" associated with watersheds, not political boundaries. The unique rules or management plan for each zone would apply to activities within it, including aquaculture facilities, recreational boating, wild baitfish harvest, etc. Zone 1, considered high risk of VHSV introduction, would be subjected to current regulatory policy with increased educational outreach. Additional precautions, such as limiting the movement of fish out of Zone 1, including those harvested from the wild or farm raised in high risk waters, should be considered. Zone 2, associated with low-minimal risk, would define a

probationary zone where activities demonstrating freedom of disease and proactive biosecurity programs could gain regulatory privileges such as fewer fish needed for testing or less frequent fish health inspections. Educational outreach to the general public should continue, however the effort should be focused on waterbodies with high boat traffic likely to move from Zone 1. Zone 3, considered protected from the introduction of VHSV, would benefit from this classification and immediately be subjected to fewer regulatory VHSV requirements.

Figure E2. Proposed zonation for risk-based management of viral hemorrhagic septicemia virus in Minnesota.



Much of the aquaculture industry in Minnesota resides in Zones 2 and 3 of this scenario and would, perhaps deservedly so, benefit from relaxed VHSV management regulations. This approach would also incentivize the aquaculture industry, promoting the implementation of biosecurity programs and voluntary testing to demonstrate freedom of disease, this in turn, would help to prevent the introduction and dissemination of VHSV in Minnesota.

E.3 Future Direction

The results of this dissertation have significantly improved the detection and management of VHS. However, given its virulence, high adaptability, and elusive nature, VHSV remains a significant threat to farm raised and wild fish populations. Only with continued efforts to understand this virus can management strategies keep pace with the virus.

Proactive surveillance by rapid and sensitive diagnostic assays (i.e. rRT-PCR) is not only recommended, but a required component of effective management. Early identification of infected populations is essential to rapidly respond, prevent an outbreak, and minimize further dissemination of VHSV. As infected populations are identified, routine phylogenetic analyses will be required to determine genetic distribution and evolution of the virus. Furthermore, these data must then be applied to ensure precise diagnostic assays, in particular rRT-PCR which relies on accurate genetic data. Finally, surveying fish populations for previous exposure to VHSV using antibody-based assays

would better inform managers of the long-term persistence and ecology of VHSV in the natural environment.

Development of additional diagnostic tests, or modification of existing assays, must also be the focus of considerable attention. Current protocols require lethal sampling, which is not only time consuming, but costly in terms of life and money. Lethal sampling is particularly challenging for hatcheries maintaining broodstock over extended periods of time and for small fish populations. Non-lethal sampling methods could include serum and water filtration. The latter is of specific interest as it would reduce the number of tests and increase the representation of the entire population. While not a new methodology, loop-mediated amplification (LAMP) assays are becoming increasingly popular for field diagnostics and would be ideal for rapid on-site hatchery screening.

Management strategies must continue to be evaluated and justified based on current science and stakeholder opinion. Implementation of the proposed risk-based management strategy will need to be performed by the state regulatory agency; however, all stakeholder opinions must be considered. Continued communication between management, industry, and researchers will ensure that the need to effectively and efficiently manage VHS is met.

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APPENDIX 1

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Aaron

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