Surveillance Proposal for Viral Hemorrhagic Septicemia Virus In Freshwater Fish in Canada and the United States

VERSION 1.0

Bilateral VHSV Surveillance Working Group:
Canadian Food Inspection Agency, Aquatic Animal Health Division
Great Lakes Fish Health Committee
U. S. Department of Agriculture Animal and Plant Health Inspection Service
U.S. Fish and Wildlife Service

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Executive Summary

Viral hemorrhagic septicemia (VHS) is a World Organization for Animal Health (OIE)-listed disease. The recent emergence of a new strain of VHS virus (VHSV) in freshwater fish in the Great Lakes region prompted the Canadian Food Inspection Agency (CFIA), U.S. Department of Agriculture (USDA) and U.S. Fish and Wildlife Service (USFWS) to undertake the collaborative development of a bilateral VHSV surveillance plan. A working group, including representatives from CFIA, the Great Lakes Fish Health Committee (GLFHC), USDA's Animal and Plant Health Inspection Service (APHIS), and USFWS was convened to structure a surveillance approach to support risk assessment and management decisions for freshwater fish culture facilities and natural freshwater systems of both the United States and Canada. The scope of diagnostics and reporting includes any strain of VHSV, but site and sample selection efforts will initially target the genotype of recent emergence in the Great Lakes, VHSV IVb. This proposal is consistent with OIE guidelines on surveillance for disease freedom as outlined in the Manual of Diagnostic Tests for Aquatic Animals, 2006. A summary of OIE guidelines to establish disease freedom is outlined in Appendix 1. For surveillance purposes, VHSV and VHS, the disease caused by the virus, are considered synonymous.

VHSV IV is considered endemic in certain marine populations of fish along the Pacific and northern Atlantic coasts of North America. However, a genotype of VHSV IV, (referred to as IVb), was recently isolated from freshwater fish associated with fish kills in the Great Lakes, an extensive watershed shared by the United States and Canada. A growing number of freshwater species (24 to date) are now considered susceptible to natural infection or disease caused by VHSV IVb (USDA VHSV IVb-Susceptible Species List 03/30/07), including many species important to recreational or commercial fisheries. Freshwater fish culture facilities at risk, should virus distribution involve farmed populations, include government and public hatcheries, as well as a variety of private commercial operations. Because live freshwater fish are moved extensively for stock enhancement, broodstock, bait, human consumption, and feed purposes, current and future VHSV IVb distribution could potentially extend well beyond the Great Lakes.

Implementation of a bilateral (Canadian - U.S.) surveillance program to establish areas of disease freedom is important to support science-based and acceptable trading conditions for live fish and certain products (other than canned fish, fish leather, and fish products destined for human consumption that have been chemically preserved, heat-treated, eviscerated, or processed into fillets or cutlets). Regulatory agencies at many levels in the U.S. and Canada also have a vested interest in protecting aquatic animal health by minimizing the spread of this virus, and of the disease it causes, throughout the freshwater aquatic ecosystem.

The goals of this surveillance effort are to efficiently and effectively: (1) determine the current distribution of VHSV IVb in both cultured and wild susceptible freshwater fish populations of the United States and Canada, (2) designate free and infected zones to facilitate disease control, and (3) implement a surveillance framework to facilitate detections of future VHSV IVb

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outbreaks. This initial surveillance effort will occur over a 2-year period. Ongoing surveillance design will be based upon the evaluation of the initial survey and resultant disease status.

The use of 'zonation' is an integral concept for this surveillance program. Surveillance zones will be determined and recorded by the Competent Authorities. Zones will be defined initially by watershed for wild fish populations; and by watershed (Canada) or administrative boundaries (United States), or both (either country), for fish culture facilities. Zones should be distinct spatial entities, separated by existing regulatory and/or geographic barriers that obstruct virus exchange. For this surveillance plan, zones are defined as 4-digit Hydrologic Unit Code (HUC-4) watersheds in the United States and secondary watersheds in Canada; or as States or Provinces, depending on the population to be sampled and the regulatory infrastructure for any planned disease control measures. Appendix 2 shows maps of the watershed zones in the United States and Canada. The U.S. and Canada share zones that drain into the Pacific Ocean, primarily through the Columbia River drainage; the Gulf of Mexico, through the Missouri River drainage; the Arctic Ocean through shared waters with Alaska; Hudson Bay through Red and Rainy River drainages; and the Atlantic Ocean, through the Great Lakes drainage.

This surveillance plan will draw conclusions about disease status to the HUC-4 or secondary watershed level. Under this plan, if each HUC-4 or secondary watershed is investigated independently as proposed, disease designations derived from surveillance findings will not need to extend to neighboring or higher-order watersheds. However, regulatory infrastructure may require that the administrative boundary takes precedent over the surveillance boundary in the interpretation of surveillance results. For example, in the U.S., existing Federal regulations restricting the movement of VHSV-susceptible species will require that the status of an infected HUC-4 apply to the entire State for activities pertaining to the Federal Order or interim rule.

Laboratory testing of fish sampled for surveillance purposes will be based on virus isolation by cell culture with subsequent confirmation of positive findings by reverse-transcriptase polymerase chain reaction (RT-PCR). The screening techniques used will detect and differentiate all known strains of VHSV. Tests will be conducted in federally-approved (APHIS or USFWS in the United States; Fisheries and Oceans Canada (DFO) in Canada), or State-recognized (U.S. only) laboratories, using harmonized protocols established through joint U.S. and Canada collaboration. Fish sampled from U.S. fish culture facilities will be selected and submitted by APHIS VS Area office, APHIS-accredited or State veterinarians. Wild fish sampled from natural watersheds will be selected and submitted by State-recognized fish health authorities (e.g., APHIS-accredited veterinarian, American Fisheries Society-certified biologist, or Federal- or State- designated employee). States may propose an alternative set of professional requirements necessary for VHSV fish health investigations in that State, e.g., for State or Federal hatcheries, or if sampling needs exceed capacity. These criteria should be documented, and available for review by other States. In all cases, sampling for confirmatory purposes of commercial farm-raised populations will require veterinary submission as described above. In Canada, fish will be collected and sampled under the auspices of the CFIA (lead agency), the appropriate Provincial authority and Fisheries and Oceans Canada.

Field validation of alternative diagnostics may allow eventual adoption of new testing modalities. Canada has been tasked with validation of the precision and accuracy of virus

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isolation and RT-PCR, and a semi-quantitative real-time PCR method (qRT-PCR). Pilot projects will explore the utility of active observational surveillance as a field screening tool to direct laboratory testing to clinical settings in future surveillance.

This surveillance proposal accommodates multiple streams of evidence on VHSV status. A central source of information is field surveillance to detect and isolate VHSV in wild and cultured populations of fish. Targeted selection is advocated over random selection of sampling units for field surveillance for VHS. Targeted selection of sites (e.g., by mortality event location, final location in drainage system, and history of live fish imports from infected regions) and fish (e.g., by susceptible species, clinical appearance and life stage) can increase the efficiency and minimize costs of surveillance by focusing efforts on presumed higher prevalence or higher susceptibility strata. However, the proposed plan also incorporates expert opinion-derived information on contextual and historical risk factors. Risk factors will help direct surveillance resources to HUC-4 and secondary watersheds with the greatest risk of VHSV IVb infection. An algorithm to determine the longevity of value of prior years' surveillance data will also result from information provided by the expert panel. Risk factors and historical data will supplement test-based surveillance data in quantitative evaluations of zone disease status.

Wild and cultured populations will be surveyed separately. VHSV IVb distribution among wild populations will be evaluated through field surveillance of a representative selection of watershed subunits (tertiary watersheds in Canada and HUC-8 watersheds in the U.S.) from each of the HUC-4 and secondary watersheds of interest. However, the ability to detect virus in wild populations in open watersheds can be limited by resource constraints and epidemiologic complexities associated with dynamic systems. Incorporating knowledge of contextual risk factors associated with the surrounding environment will help to (1) direct field surveillance efforts to watersheds with the greatest VHSV IVb-status uncertainty and (2) supplement field data where surveillance funds are limited. To do this, risk factors (e.g., presence of susceptible species, hydrologic connectivity with known infected sites, or previous use for culture or enhancement activities) will be weighted as predictors for freshwater VHSV IVb infection by a designated panel of experts. The risk score for a given watershed or zone can then be combined with field surveillance results through a simple (odds form) Bayesian model to calculate the presumed (posterior) probability of VHSV IVb infection. The resulting decision metric targets the need for additional field surveillance and builds the case for disease freedom using available systematic and complex data streams.

Evaluation of VHSV distribution among fish culture facilities will parallel the process in wild populations. In Canada, definitions differentiating types of culture facilities are in progress. In the U.S., for the purpose of surveillance design, cultured fish facilities include farm-raised or farm-managed populations. However, regulatory or compensation decisions that result from surveillance findings in a culture facility may differ between farm-raised vs. farm-managed populations and according to available biosecurity measures. For surveillance purposes, fish culture facilities can be grouped by watershed or by State or Province, or evaluated on an individual basis. Where establishments are grouped by administrative boundary, zones will be prioritized for field surveillance by the highest risk score designated to watersheds contained within, traversing or abutting the zone. In the US, participating States will provide a registry of fish culture facilities with VHSV-susceptible species that are (1) currently or historically

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involved in live fish sales, exchange or stock enhancement with any other zones, and (2) willing (or State-mandated) to participate in VHSV surveillance. A set of facilities will be randomly selected from each registry to determine surveillance locations for cultured populations. Negative field surveillance results, combined with administrative management of zone biosecurity, will support disease freedom claims for registered fish culture facilities in regions associated with low-risk watersheds. Establishing disease-free compartments (e.g., by species or facility), per OIE guidelines, in zones associated with high-risk or VHSV IVb positive watersheds could potentially facilitate movement from high-risk regions.

This surveillance plan enables disease status conclusions to be drawn to the secondary (Canada) and HUC-4 (U.S.) watershed level. Results from this surveillance initiative will support VHSV IVb disease freedom claims for designated zones or compartments. Results will also guide risk-based management or regulatory decisions where disease freedom status has not been achieved. VHSV IVb probability calculations for open watersheds will be revised annually, discounting historical data by introduction risk, incorporating new findings, and re-visiting risk factors as needed. Zones with negative test results sufficient to support a VHSV IVb freedom claim can maintain that status through ongoing surveillance and demonstration of effective biosecurity, and/or consistently low calculated VHSV IVb probabilities. Alternative screening modalities, such as active observational surveillance, may provide options to minimize costs of ongoing surveillance. Results from this VHSV IVb surveillance could be generalized to other strains of VHSV in tested watersheds, although system sensitivity for other strains may vary with targeting criteria. Specific protocols for ongoing surveillance, including expert panel revision of risk factors, alternative screening modalities, and review of algorithms for incorporating historical data, will be detailed following a performance review of the first 2 years of surveillance.

Communications (brochures, websites, manuals, presentations and workshops) about this surveillance plan, and regulatory and/or infrastructural advantages participation will provide, will help garner support from stakeholders bilaterally. Their participation is essential for the success of this plan. Funding to build the fish health infrastructure at the State/Provincial and local levels, provide the technical training, manage the database and conduct surveillance diagnostics is also essential to this plan's success. In addition, public communications are important to raise awareness about zonal biosecurity measures necessary to control disease spread. The raised public and stakeholder awareness of fish health and biosecurity, and the enhanced collaborative infrastructure for fish health surveillance, will advance detection and response to VHS and future aquatic animal diseases both in internationally-shared and domestic resources.

An overview of the surveillance plan is provided in the next section to orient the reader to the requirements of the plan. Description and justification for each surveillance step follows in the central document. Sections I and II outline guiding principles and required infrastructure. Sections III through VI detail the proposed surveillance strategy. Sections VII and VIII outline implementation and evaluation components. Term definitions and the VHSV IVb classification system (e.g., technical definitions of VHSV IVb-free, suspect, unclassified, and infected) are listed in Sections IX and X. It is recommended that readers familiarize themselves with the definitions and classification system as they are specific to this document. Further description of certain plan components or methods is detailed in technical appendices of Section XI.

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Surveillance Overview

This section provides a brief outline of the steps required to demonstrate freedom from disease, or assess probability of infection, for a given State, province or watershed. Details and justification for steps listed in Table 1 (Initial Surveillance) are provided in later sections of this proposal. Details for Table 2 (Ongoing Surveillance) will be provided in a separate document pending outcomes of the review of the initial surveillance period.

Surveillance will be conducted in parallel in wild and cultured populations. The first 2 years of surveillance will generate data for the initial categorization of zones as infected, unclassified or free. Ongoing surveillance will then proceed following a collaborative evaluation of the initial surveillance program in Canada and the United States. An outline of the steps required for each surveillance period (initial and ongoing) in each population segment (cultured and wild) is presented in Tables 1 and 2 below.

Table 1: Summary of steps involved in the initial VHSV surveillance period, described separately for wild and cultured fish populations. Supporting details for each step are provided in later sections of this manuscript.

	Initial Surveillance (0-2 years)
Wild fish surveillance[1]	**a. Establish zones for wild fish surveillance**[1]**.** Establish spatial zones using watershed boundaries. These zones will initially be delineated by 4-digit HUC for the U.S. and secondary watersheds for Canada. If disease freedom claims are to be made, zones should meet OIE guidelines and Competent Authority approval. **b. Estimate VHSV IVb infection risk.** Estimate VHSV IVb risk for each zone based on expert-derived risk factor weights. Use risk score to prioritize resources for field surveillance across zones. Higher scoring zones should receive greater surveillance intensity. **c. Construct sampling frames.** For zones considered more than negligible risk, create a list frame of constituent geographic subunits (water bodies) based on 8-digit HUC delineations for the U.S. and tertiary watersheds in Canada. Where necessary to produce sufficient numbers of subunits for sampling, further subdivisions can be created using grid systems determined by the State or Province. These subunits will be considered sampling units for surveillance purposes. **d. Prioritize surveillance effort.** The number of surveillance sites necessary for disease freedom investigation of a particular zone will vary by risk of infection[2] and can be determined from Table 1.1. Zones scored negligible VHSV-risk will not require formal field surveillance. Zones already known to be VHSV-infected will also not be prioritized for wild population surveillance. For all other scores, the ideal number of sampling units (subunit sites) for field surveillance can be determined from the 10% detection threshold column below. Note that this value is based on the premise that VHSV is highly contagious and likely to rapidly spread (to at least 10% of subunits) throughout an infected watershed system. A more conservative assumption (spread to only 2% of subunits) would yield higher sample sizes (2% detection threshold column). Prior surveillance data may be retained, to reduce site numbers required in subsequent assessments, if properly discounted by period risk of VHSV introduction.

[1] Surveillance of wild fish will include wild (free-ranging) populations in the U.S. and both wild fish (free-ranging) populations and public fish culture facility (e.g., government hatchery) populations in Canada.

[2] Risk of infection is derived from the results of an expert panel tasked with weighting factors of perceived importance to VHSV infection. A risk score is the product of likelihood ratios assigned to factors relevant to a given watershed. The expert panel derivation of risk scores is currently in process, with an expected completion date of July 2007.

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Initial Surveillance (0-2 years)

Table 1.1: Risk-adjusted number of subunits (e.g., HUC-8 or tertiary watersheds) recommended for initial surveillance to determine disease freedom for a specific (HUC-4 or secondary) watershed[3]. These calculated sample sizes presume 95% confidence, 95% sensitivity and 100% specificity, and are derived for list-frames totaling 100 subunits, or less, in size.

Risk Category	Risk Score (x)	Surveillance for 10% detection threshold	Surveillance for 2% detection threshold
Negligible	≤ 0.01	None	None
	0.01 < x ≤ 0.02	None	5 subunits
Low	0.02 < x ≤ 0.1	5 subunits	16 subunits
Moderate	0.1 < x ≤ 0.25	10 subunits	36 subunits
	0.25 < x ≤ 0.5	20 subunits	55 subunits
	0.5 < x ≤ 1	30 subunits	82 subunits
High	> 1	≥ 30 subunits	≥ 82 subunits

e. Target subunits for sampling. Select either (1) ≥ 30 subunits, or (2) a lesser subset determined by risk-score (see above). Targeted selection of subunits by mortality event location, drainage patterns (e.g., final drainage), history of culture or stock enhancement activities, or angling pressure and live bait use is recommended.

f. Choose capture location(s) and methodology. Within each selected subunit (e.g., HUC-8 or tertiary watershed), harvest fish using best available technology and local knowledge of susceptible species distributions, abundance and seasonal congregations (e.g., for spawning).

g. Target fish for collection. During the spring or fall (for 2 years), collect a total of 170 fish from each selected watershed subunit. Fish should be targeted by tier group (Appendix 3) for susceptible species (excluding endangered or threatened populations), clinical appearance (targeting moribunds if possible) and recent post-hatch or reproductive age-class. Systematic selection should be used for apparently healthy fish. In an outbreak investigation, if moribund fish are readily obtained, sample sizes can be reduced to 35 moribund fish (from the assemblage of susceptible species) per investigation.

h. Submit samples to approved laboratory. Submit whole fish or tissues (kidney, spleen and/or heart) from sampled fish whenever possible. Lethal sampling of declining populations should be avoided. Ovarian fluid (but not milt) may be substituted for lethal sampling in these situations.

i. Calculate posterior probability of infection. Combine field surveillance results with expert opinion-based risk factor knowledge, by Bayesian model, to estimate VHSV IVb infection probability for each watershed.

j. Evaluate disease freedom. Negative results from this surveillance are sufficient to claim tentative VHSV IVb freedom (95% confidence) at a 10% site-level detection threshold for the watershed (and 5% design prevalence within a fish population). However, watersheds must be recognized as biosecure zones by Competent Authority, and/or institute an ongoing surveillance program, to effectively maintain that status.

k. Optional field validation. Conduct a pilot study of active observational surveillance (AOS), employed at the subunit level, as a diagnostic screening tool to direct (and minimize) laboratory testing for ongoing surveillance. Parallel testing of randomly selected locations/dates would provide data for AOS field validation.

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[3] These sample size requirements are derived from Bayes' theorem for posterior odds of infection targeting a resultant site-level probability of infection below 10% (or 2%, last column). Solving for prior odds of infection, given selected risk scores (derived from the product of applicable risk factor likelihood ratios) provides a target probability of infection (probability=odds/(1+odds)) that needs to be generated from field surveillance activities in order to provide suitable confidence in disease freedom. The sample sizes (of surveillance sites) necessary to generate that target probability can be determined using FreeCalc[5].

Initial Surveillance (0-2 years)

Cultured Fish surveillance[4]

a. Establish zones for cultured fish surveillance.[4] Zones should meet OIE guidelines for administrative oversight of biosecurity and be accepted by Competent Authorities. Default zones will follow State boundaries in the U.S.

b. Estimate VHSV IVb risk. Estimate VHSV IVb risk for each watershed in the State based on expert-derived risk factor weights. Use the highest watershed risk score in each State to prioritize resources for field surveillance across States. Higher scoring States (zones) should receive greater surveillance intensity.

c. Create sampling frames. Obtain a registry, from each State (zone), of fish culture facilities with susceptible species that are (1) currently or historically involved in live fish sales, exchange or stock enhancement with any other zones, and (2) willing (or state-mandated) to participate in VHSV surveillance.

d. Prioritize surveillance effort to higher-risk States. States (zones) scored negligible VHSV-risk would not require formal field surveillance. For all other States (including States with known-infected watersheds) choose the number of establishments required for a 2% detection threshold, given the highest risk scored to State waters. The calculated sample sizes shown below presume 95% confidence, 95% sensitivity and 100% specificity, and are derived for list-frames totaling 2,000 farms or fewer in size. Prior surveillance data may be retained, to reduce site numbers required in subsequent assessments, if properly discounted by period risk of VHSV introduction.

Table 1.2: Risk-adjusted number of fish culture facilities recommended for initial surveillance to determine disease freedom for a specific State or zone.

Highest Watershed Risk Category in State	Risk Factor Score (x)	Number of fish culture facilities requiring surveillance, per total on State registry					
		30 total	50 total	100 total	200 total	500 total	2000 total
Negligible	≤ 0.01	None	None	None	None	None	None
	0.01 < x ≤ 0.02	5	5	5	5	5	5
Low	0.02 < x ≤ 0.1	14	16	16	17	17	17
Moderate	0.1 < x ≤ 0.25	25	27	36	40	42	44
	0.25 < x ≤ 0.5	30	41	55	65	72	76
	0.5 < x ≤ 1	30	50	82	111	136	151
High	> 1	30	50	≥ 82	≥ 111	≥ 136	≥ 151

e. Select sites at random for surveillance. Use a random numbers table or software to select the recommended number of facilities from each State registry for surveillance.

f. Conduct veterinary inspections. Twice a year (spring and fall), for 2 years, conduct a veterinary inspection of all holdings (ponds, tanks, raceways) on selected establishments. Prioritize any high-risk holdings (e.g., recurrent imports, mixed lots, final recipient of shared water, recent mortality or clinical disease) for collection.

g. Target fish for testing. Collect a representative sample of fish from each VHSV-susceptible population (grouped by species and year-class) for laboratory testing. Populations should comprise a single year-class and species, rather than containment (which can vary widely in definition and type). During veterinary inspections, target moribund fish, if possible. For species managed as single year-classes, select 35 moribund, or 70 'random' fish from each year-class/species. These numbers assume a design prevalence of 5% across

[4] Surveillance of cultured fish populations will include public and private fish culture facilities (including government hatchery) in the U.S., and private establishments on a volunteer basis in Canada. Both U.S. farm-raised and farm-managed fish populations will follow the same (cultured fish populations) surveillance protocols. However, U.S. farm-raised and farm-managed populations may fall under different regulatory and compensation requirements.

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Initial Surveillance (0-2 years)	
	the general population, population sizes exceeding 500 fish, 95% confidence, 85% sensitivity and 100% specificity. FreeCalc[5] can be used to determine sample sizes required of smaller populations. For species managed as mixed-lots, including baitfish routinely harvested from more than one location and occasion, collect a representative sample of 170 fish (comprised of VHSV susceptible species) from each mixed-lot. Movement testing data can be used for surveillance purposes if it meets all specifications described above. **h. Submit samples to an approved laboratory.** Submit whole fish or tissues (kidney, spleen, and/or heart) from sacrificed fish whenever possible. Ovarian fluids (but not milt) may be substituted if sacrifice of healthy broodstock is not practical or economically feasible. **i. Evaluate disease freedom**. Negative results from this surveillance would be sufficient to claim (at a 95% confidence level) VHSV freedom at 2% design prevalence for registered fish culture facilities within the zone (and 5% design prevalence within each fish population). If associated watersheds are also considered low risk (see below), the zone itself can be claimed VHSV-free. **j. Optional field validation.** Conduct a pilot study of active observational surveillance (AOS), employed by interested establishments, as a diagnostic screening tool to direct (and minimize) laboratory-testing for ongoing surveillance. Parallel testing of randomly selected containments/fish within AOS establishments would provide data for AOS field validation.

Table 2: Summary of steps involved in ongoing VHSV surveillance, described jointly for both cultured and wild populations. Specific details will be provided following a performance review of the initial surveillance period.

Ongoing Surveillance (after initial 2 years)	
Surveillance in disease-free zones that can demonstrate effective biosecurity	a. Demonstrate biosecurity, per national and/or State/Provincial guidelines, for zones claiming disease freedom. b. Annually update (Bayesian model) probability of VHSV IVb infection for associated watersheds. Presumably biosecurity would ensure low risk of introduction and minimal need for continued testing. c. Demonstrate infrastructure for detecting/reporting new VHSV outbreaks within the zone. A functional AOS could provide this assurance.
Surveillance in unclassified zones or disease-free regions without effective biosecurity	a. Annually update (Bayesian model) probability of VHSV IVb infection for associated watersheds. Surveillance data from previous years, discounted by the risk of new introduction, can be used as an informed prior for probability distributions generated from new field data. b. Annually sample fish culture facilities to improve or maintain confidence in negative status. b. Implement a validated AOS to supplement field-testing requirements aiming to demonstrate or maintain disease-freedom status.
Surveillance in infected zones	a. If eradication is possible, conduct field surveillance (as described for initial surveillance) for ≥2 years to build evidence of disease freedom. b. If eradication is not possible, consider compartmentalization, per OIE and Competent Authority guidelines, of VHSV-free fish culture facilities, or groups of fish culture facilities, or other defensibly biosecure subunits within the zone.

[5] FreeCalc Version 2 [http://www.ausvet.com.au/content.php?page=res_software] can be used to calculate sample sizes for number of sites and number of fish to sample within each site.

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I. Introduction

A. Disease Description

Viral hemorrhagic septicemia virus (VHSV) is a rhabdovirus that infects a wide range of marine, brackish and freshwater fish species, including anadromous species such as many salmonids. To date, the virus has been found in over 65 different fish species. Four major genogroups of VHSV have been identified (Einer-Jensen et al., 2004; Snow et al., 2004) Genogroup I consists mainly of freshwater strains isolated in Europe, although it has been isolated from marine fish around Europe and rainbow trout raised in brackish water around Finland. Genogroups II and III are endemic in wild marine fish found around Europe. Genogroup III has been isolated from Greenland Flounder caught at the Flemish Cap. Genogroup IV is considered endemic in certain populations of wild marine fish along the Pacific coast of North America (subgroups IVa) and in Japan. A genotype of IV was isolated from mummichogs, sticklebacks, striped bass and sea-run brown trout on the Atlantic coast of Canada (Gagné et al., 2007). This genotype has not yet been named. Recently, in 2003, 2005, 2006 and 2007, another genotype of IV distinct from other sequenced North American (east and west coast) VHSV IV isolates has emerged in free-ranging freshwater species of the Great Lakes region (Elsayed et al., 2006). This genotype has been named IVb. Multiple large die-offs in the Great Lakes region involving at least 14 different species, to date, have been attributed to VSHV IVb since the spring of 2006 (Michigan DNR briefing paper). The virus has also been found in inland lakes in several States, including a lake in Wisconsin that is part of an all-water route between the Great Lakes and the Mississippi River. To date, this freshwater IVb genotype has not been found in fish culture facilities. However, its emergence in natural freshwater systems has heightened concerns over its potential impacts to freshwater fish culture facilities and wild populations throughout the United States and Canada. Large-scale fish movements between Lakes Huron and Michigan (Michigan DNR briefing paper) and water passage into Lake Superior and the Mississippi drainage suggest the potential for natural expansion of the range of the virus. Extensive anthropogenic movements of fish and fomites (e.g., by anglers, ballast water, stock enhancement or commercial trade) extend the potential range beyond existing hydrographic boundaries.

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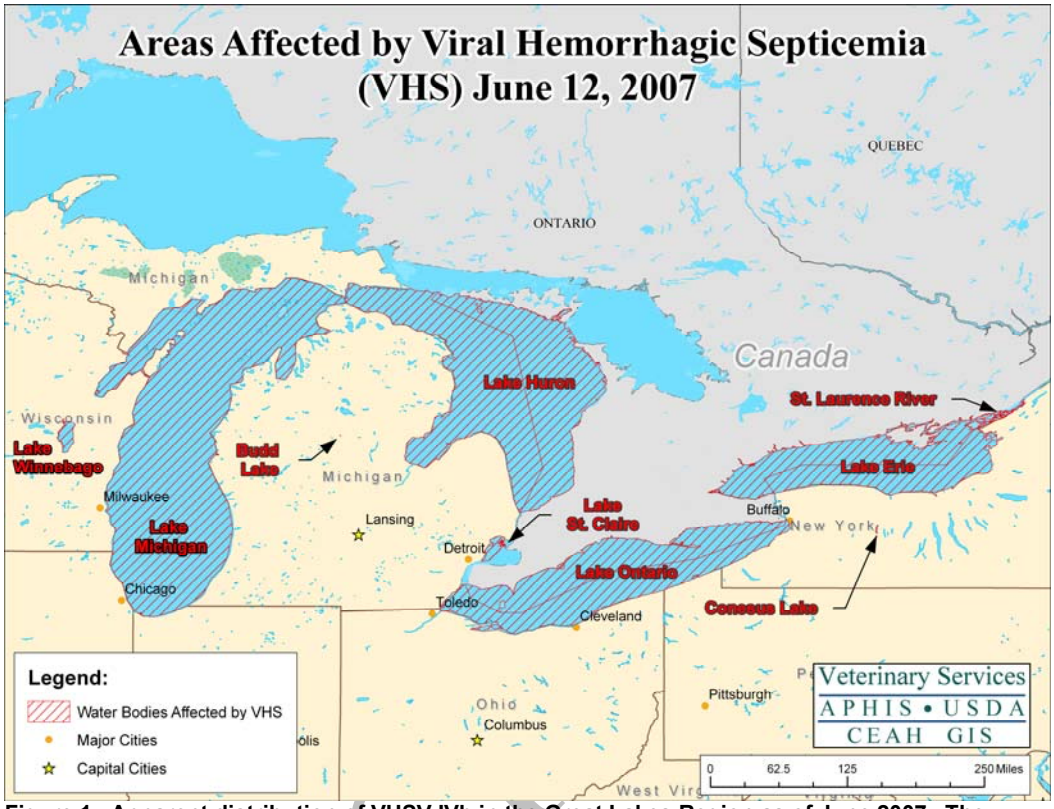

Figure 1. Apparent distribution of VHSV IVb in the Great Lakes Region as of June 2007. The recent findings in inland lakes of Wisconsin and Michigan, as well as Lake Michigan, are suspect positives, still in the process of final confirmation.

The pathogenicity of VHSV varies with genotype, fish species and environmental factors. For instance, Pacific isolates of VSHV IVa are highly pathogenic to Pacific herring, but these isolates have experimentally caused little or no mortality in rainbow trout reared in freshwater. Age can also influence susceptibility to VHSV; younger fish are more likely to become infected and develop disease (International Aquatic DB). VHSV shed by infected fish through excretory products, predominantly urine, may remain viable in water for weeks depending on presence of organic matter, water temperature, and original titer. Virus introduction to naive watersheds is presumed to occur by wild fish movements, and live fish transfers for angling, commercial or stock enhancement purposes (Skall et al., 2005). Consequently, virus may readily distribute throughout an infected watershed via the natural migrations of resident fish or normal hydrologic flow of water. Movement of contaminated ballast water and fomites such as boats and equipment are other speculated potential sources of virus introduction. Fish-eating birds and possibly parasites may also play a role in the transfer of viruses among aquatic populations (Peters and Neukirch, 1986).

Water temperature may influence the infectivity of VHSV. Morbidity and mortality associated with VHSV infection is commonly noted at water temperatures between approximately 40°F and

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57° F (4°C and 14°C), although viral growth has been documented as high as 25°C. Although outbreaks of disease can arise throughout the year, changing water temperatures in the spring and fall, coupled with the dynamics of the immune system of poikilotherms, and other predisposing factors, such as poor nutrition or parasitic infestations, may create optimal conditions for disease occurrence (International Aquatic DB).

Clinical signs of VHSV include: hemorrhage in the skin, especially on the lateral or ventral surfaces or anterior portion of the head; exophthalmia; ascites, and congested or hemorrhagic organs including liver, spleen, kidney, intestines, heart, meninges of the brain, or swim bladder. Fish often appear listless, swim in circles or float just beneath the surface. Mortality rates for Genotype IVb (freshwater strain) are unknown, but for Genotype I, it can vary from 80-100 percent in young fish to 10-50 percent in older fish.

B. Rationale for Surveillance

The further emergence of VHSV throughout freshwater systems of the United States and Canada could substantially impact major aquatic commodities, such as baitfish, catfish and salmonids. The common large scale movement of gametes, embryos, fish, and water between water bodies, watersheds, and enhancement and commercially farmed fish facilities threatens to transmit VHSV IVb into previously free areas. Surveillance and subsequent regulatory action has been recommended to define the distribution of VHSV IVb and to help control future spread of this emerging disease in freshwater systems.

In 2005, the value of the entire U.S. aquaculture industry totaled $1.092 billion USD. The economic value of the VHSV IVb susceptible species in commercial aquaculture, as reported in the 2005 Census of Aquaculture, totaled approximately $613 million USD. Data for all species were not available, so this number is likely overestimated since certain categories of fish were aggregated. Production, as measured by value of sales, from the five States bordering the lower Great Lakes (New York, Pennsylvania, Michigan, Ohio, and Wisconsin) comprised 2 percent of the total value of U.S. food fish production, over 13 percent of baitfish, and approximately 8 percent of the sport/game fish industry in 2005 (NASS, October 2006). Commercial wild fish landings of VHSV susceptible species in these 5 States, reported as sold during 2005, totaled approximately $12 million (NMFS commercial landings database[6]).

In Canada, Ontario's live bait fish industry generated over $14 million CDN in 2005 from a harvest of approximately 8.7 million dozen fish (Ontario Ministry of Natural Resources, 2005). Approximately 1 million dozen bait fish were exported from Ontario. In 2000, the commercial fishery in the Canadian portion of the Great Lakes was valued at approximately $43 million CDN (on approximately 31 million lbs of fish) and consisted of yellow perch, lake whitefish, walleye, chubs, smelt, lake trout, channel catfish, and carp (Kinnunen, 2003). Some of these species are also susceptible to infection by VHSV IVb.

C. Purpose of VHSV Surveillance

The purpose of VHSV surveillance is multifold and includes the following goals.

- Describe the freshwater distribution of VHSV IVb among cultured and wild freshwater fish populations in the United States and Canada;

[6] http://www.st.nmfs.gov/st1/commercial/landings/annual_landings.html

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- Identify VHSV IVb-infected zones (whether by HUC-4 or secondary watershed, or State/Provincial boundaries) to inform disease control efforts;
- Establish disease-free zones (whether by HUC-4 or secondary watershed, or State/Provincial boundaries) to improve trading-partner confidence;
- Facilitate the early detection of VHSV in VHSV-free or VHSV-unclassified zones; and
- Collect surveillance data in a manner that informs disease control decisions, but also supports future surveillance system improvement and epidemiologic analyses.

D. VHSV Surveillance Objectives

The following objectives are the specific tasks and activities that allow for the achievement of the purpose of VHSV surveillance in freshwater systems of the United States and Canada.

- Conduct systematic diagnostic test-based freshwater surveillance of VHSV IVb susceptible fish in fish culture facilities and wild fish populations;
- Elicit subjective data from fish health experts to generate likelihood ratios for freshwater watershed VHSV IVb risk factors;
- Combine multiple streams of evidence (e.g., surveillance, historic, and expert opinion-derived risk factor data), using a Bayesian model, to estimate the probability of VHSV IVb in any given watershed or zone;
- Identify freedom from disease, with 95% confidence, for HUC-4 or secondary watersheds or geopolitical zones whose probability of VHSV IVb (by Bayesian model) is ≤ 10% among watershed subunits and ≤ 2% among fish culture facilities (and ≤ 5% among fish within an assemblage or facility);
- Map the occurrence of all strains of VHSV detected in US and Canadian watersheds;
- Pilot test active observational surveillance in a volunteer subset of fish culture facilities and wild fish populations;
- Field-validate the VHSV detection accuracy of viral isolation followed by RT-PCR;
- Field-validate alternative VHSV screening methods (e.g., quantitative or real-time RT-PCR, and active observational surveillance) that might improve surveillance practicality, sensitivity or cost in future applications.

E. Expected Outcomes

- Disease freedom status will be documented for zones and compartments meeting statistical and risk criteria, and biosecurity requirements;
- A flexible approach will be developed for comparing the probability of VHSV IVb infection across all HUC-4 and secondary watersheds through a combination of survey results and expert-derived scoring of risk factors;
- Field validation will provide estimates of the accuracy of alternative screening modalities, (e.g., qRT-PCR, and active observational surveillance); and
- An enhanced collaborative infrastructure for fish health surveillance will facilitate future detection and response to VHS and other aquatic animal diseases in shared and domestic resources.

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II. VHSV Surveillance Infrastructure

A. Stakeholders

The stakeholder list includes (but is not limited to) recreational fishing industries, anglers, commercial fish producers, commercial fishermen, U.S. federal agencies, State-level conservation, natural resource management, fish/game/wildlife and agriculture agencies, Canadian Departments and Ministries, other freshwater fish associations, First Nations (Tribal Nations), aquarium and ornamental fish industries, diagnostic laboratories, researchers, regional commissions and private citizens.

B. Responsible Parties

1. Bilateral VHSV Surveillance Committee

A bilateral VHSV Surveillance Committee will be established to consider any necessary revisions or modifications to the existing surveillance protocols. The committee will consist of representatives from USDA APHIS, CFIA, USFWS, DFO, State and Provincial fish and wildlife or natural resources and agricultural ministries, and GLFHC. Other members will be identified as required. The committee will meet at least once annually.

2. Fish Health Inspectors

In Canada, all fish sampling will be under the auspices of the CFIA. Wild fish will be sampled by Federal or Provincial employees or designates. Fish in commercial fish culture facilities will be sampled by a licensed veterinarian or by a Federal or Provincial employee.

Fish sampled from U.S. fish culture facilities will be selected and submitted by APHIS VS Area office, APHIS-accredited or State veterinarians. Wild fish sampled from natural watersheds will be selected and submitted by State-recognized fish health authorities (e.g., APHIS-accredited veterinarian, American Fisheries Society-certified biologist, or Federal- or State- designated employee). States may propose an alternative set of professional requirements necessary for VHSV fish health investigations in that State, e.g., for State or Federal hatcheries, or if sampling needs exceed capacity. These criteria should be documented, and available for review. In all cases, sampling for confirmatory purposes of commercial farm-raised populations will require veterinary submission as described above. Training for general disease recognition and knowledge of VHSV clinical signs, site inspection and fish handling/sampling procedures will be provided through an APHIS- or AFS-approved course.

Site inspections of cultured or managed fish facilities will include a farm visit, visual inspection of fish in component systems for clinical signs of disease, collection of suitable fish for diagnostic testing and necropsy, recording of positive and negative observations (e.g., clinical, gross and historical findings) as well as species, date/temperature and identification of systems examined. Review and consultation of site biosecurity, disease monitoring and reporting protocols will facilitate efforts to maintain disease freedom. Watershed site inspectors should record the date, location, water temperature, species, numbers and methods/findings of populations examined. A standardized form for recording observations during fish culture facility and watershed site inspections will be made available prior to program implementation.

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3. Field Observers

Potential observers for observational surveillance of fish culture facilities include producers, natural resource officials (Federal, State/Provincial, tribal, and private), and fish health professionals. Potential observers for wild fish populations include, among others, natural resource officials (Federal, State/Provincial and private), creel survey clerks, charter-boat captains and registered fishing guides or fishing-tournament hosts.

4. Participating Laboratories

In the United States, APHIS- or other Federal agency-approved or State-recognized laboratories following approved and harmonized protocols may conduct testing for VHSV surveillance. In Canada, DFO laboratories, which comprise the National Aquatic Animal Health Laboratory System (NAAHLS), will be the primary laboratories responsible for the laboratory testing and validation of testing protocols for VHSV surveillance. DFO may approve other laboratories outside NAAHLS to conduct testing.

C. Sample Handling, Transport and Processing

Whole live fish, fish organs (kidney, spleen and heart), or ovarian fluid constitute the samples that will be transported to designated laboratories. Fish must be live, or fresh-dead, when sampled (Appendix 4). Samples will be shipped in the appropriate manner (Appendix 4) to the designated laboratory. The laboratory will process samples according to the methods outlined in Appendix 5. If samples cannot be processed immediately upon receipt, the laboratory will keep the samples at -80˚C for a maximum of 3 weeks. DFO's National Laboratory Manager will design validation and test operating characteristic studies for virus isolation and qRT-PCR. Samples collected for this purpose will be clearly labeled.

D. Laboratory Standards

Laboratory standards (Appendix 5) for this surveillance will be set by DFO and USDA APHIS-Veterinary Services (VS) through a joint U.S.-Canadian laboratory commission. DFO and APHIS will utilize laboratory protocols equivalent to OIE guidelines, where available, for virus isolation by cell culture, VHSV identification by RT-PCR, and VHSV strain identification through molecular genotyping. DFO will establish standard protocols for screening for VHSV using qRT-PCR. In addition, DFO and APHIS will conduct test validations with respect to precision [repeatability (within laboratory) and reproducibility (between laboratories)] in their respective countries.

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E. Outreach Education

Outreach education is required at three different levels:
1) Meetings will be held with stakeholders and State/Provincial authorities to explain the surveillance plan, garner support and assistance, and allocate responsibilities and activities.
2) Technical training will be provided for individuals designated to conduct the various components of the surveillance plan.
3) It is recommended that one or more joint U.S.-Canada web sites be created, using the National Surveillance Unit's avian influenza model, to disseminate accurate, consistent information and surveillance results across agencies and to the general public.

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III. VHSV Surveillance Methods and Assumptions

This section describes VHSV surveillance methods and assumptions that are common to both cultured and wild fish populations.

A. Surveillance Zones

The Competent Authority may define surveillance zones by watershed, administrative boundary (e.g., State or Province), or some combination of these. Zone boundaries must be clearly documented and approved by the responsible Competent Authority prior to initiation of surveillance. The zonation system sets the framework around which surveillance data are aggregated, and therefore can affect sample sizes required for disease freedom investigation. However, surveillance guidelines and procedures presented in this document otherwise apply uniformly to any of the aggregation schemes. Zones should meet biosecurity and recording requirements outlined by OIE, and agreed upon by the Competent Authorities, in order to maintain disease freedom status without continual surveillance.

B. Surveillance Data Sources

There are six data sources in this surveillance proposal. Data types include: (1) historical data, (2) demographic data, (3) expert opinion data, (4) active field surveillance based on laboratory tests, (5) active field surveillance based on observation for clinical signs or gross pathology and confirmed by laboratory tests (termed active observational surveillance, or AOS), and (6) disease outbreak investigation or movement testing data.

Historical and risk factor data will be solicited and used initially to classify VHSV risk in all zones. Surveillance efforts, in both wild and cultured populations, will be prioritized by this initial risk score. Field surveillance form the core of disease freedom investigations. However, standard disease freedom investigations may be limited by practical constraints, especially in open watershed systems. Consequently, systematic (current and historic field surveillance) and complex (expert opinion-derived risk factor) data will be combined through Bayesian model to estimate probability of VHSV-infection in a given zone. If the calculated probability of VHSV-infection is less than the target design prevalence, tentative disease freedom can be claimed. Maintaining disease freedom status would require concurrent assurance of zone biosecurity and/or ongoing surveillance. If the calculated probability of VHSV-infection is not less than the target design prevalence, the results can still guide local management and regulatory decisions based on disease risk. A user-friendly Bayesian model of VHSV probability (and conversely disease freedom) for any given zone, combining data from these various sources, will be available to participating States/Provinces. States/Provinces may request the model (and help using the model), or may request NSU/CFIA to run the model and return the calculated results.

1. Historical Data

Historical data includes results of fish health testing programs conducted prior to implementation of the bilateral VHSV surveillance plan by U.S. and Canadian Federal, State and Provincial governments; academic or other studies conducted in the United States and Canada; and fish import data. Negative historical data that do not meet specifications of the current bilateral VHSV surveillance plan may be allowed to alter the (otherwise un-

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informed, e.g., uniform) prior probability of current surveillance results. This will be determined in consultation with the expert panel (Appendix 6).

Historical data also includes results of surveillance testing that does follow the VHSV surveillance plan protocols. This prior surveillance data will be discounted by risk of introduction (A. Cameron, AusVet Animal Health Services, personal communication), e.g., per risk factors assigned by the expert panel, and retained for VHSV probability calculation in the current year. Prior data are retained by incorporation as a 'prior probability' in current surveillance-derived prevalence distributions (Appendix 6). Where the period risk of VHSV introduction is minimal, the need for new surveillance testing may be substantially reduced by allowed carryover of results from the previous period. The VHSV probability model provided to participating States/Provinces, including a provision for prior data, will automate this process.

2. Demographic Data

When possible, the following information will be collected for each watershed: number of lakes and associated rivers, natural land-locked lakes, fish culture facilities, fish movements within and between watersheds, susceptible fish species and a (quantitative or qualitative) estimate of the number of populations and their size, and any biosecurity measures (standard operating procedures, policies or regulations) in place for fish culture facilities, the watershed or the zone. These data will be used to prioritize surveillance efforts by risk (see Expert Opinion Data below) and to provide risk factor data for VHSV infection evaluations of individual watersheds.

3. Expert Opinion Data

While empirical field surveys generate critical information on the probability of watershed infection, risk factor data are also valuable. Certain contextual factors may be extremely important to comprehensive understanding of disease risk. These factors might include, *for example*, presence/absence of susceptible species or suitable water temperatures; barriers to water, fish or human movement; hydrologic proximity to infected sites; known fish migrations from infected waters; known prior shipments of fish from affected waters; unregulated movements of live fish from any jurisdiction; extent of recreational traffic; frequency of fishing tournaments; angler pressure; and degree of compliance or enforcement of fish health standards in the region. For VHSV IVb, as with many emerging diseases, empirical data on risk factors is limited. Consequently expert opinion will form the basis of an initial assessment of the predictive strength of contextual and historical factors (Appendix 6).

The numerator and denominator of a subjective likelihood ratio[7] for a risk factor can be generated by asking experts to estimate the prevalence of a risk characteristic among a hypothetical group of VHSV IVb affected watersheds, and then to estimate the prevalence

[7] Likelihood ratios provide summary measures of the reliability of risk factors as predictors of infection: a ratio of the predictor's true positive to false positive rates. A value greater or less than 1 implies diagnostic power as a risk or protective factor, respectively. A value of 1, or close to 1, suggests that the factor has limited to no predictive power. The product of the applicable likelihood ratios, or Bayes factor, for a given watershed represents the combined 'weight' of the risk factor-derived evidence of infection.

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of that same characteristic among hypothetical non-VHSV IVb affected watersheds. The range of expert responses can be used to fit a distribution and define confidence limits around estimated parameters. These weights can be used to assign a preliminary risk of VHSV in various watersheds. Following a Bayesian model format[8], the posterior probability[9] of VHSV can be calculated from the product of applicable risk factor likelihood ratios and the surveillance-derived prevalence estimate[10]. Again, this process will be automated for participating States/Provinces. Risk-adjusted sample sizes necessary for disease freedom can be determined from this same equation by solving for the surveillance-derived prevalence required, given a known risk score, to demonstrate a posterior probability of infection less than the design prevalence. The sample sizes necessary to meet a specified design prevalence are generated using FreeCalc and shown in Table 1.1.

4. Test-Based Field Surveillance

Field surveillance will follow multiple stages of sampling. Zones will be prioritized for sampling based on an expert-derived assessment of VHSV risk (Tables 1.1 and 1.2). Targeting will be used to different degrees in wild and cultured populations to guide site and/or fish selection (Figure 2). For fish culture facilities, sites will be selected at random from a registry of all facilities that are (1) participating in live fish movements to/from other zones, and (2) willing, or State-mandated, to participate in VHSV surveillance. Susceptible species will be grouped by year-class (rather than containment) for sampling purposes. Moribund or high-risk fish will then be targeted for collection by veterinary inspection. For wild populations, subunit sites will be targeted by exposure potential. The specific location(s) of collection efforts within a subunit will follow local knowledge of abundance or congregations. Wild fish (or mixed-lot) assemblages will be grouped by collection event, rather than species, for sampling purposes. Highest susceptibility species, clinical appearance and age-class fish will then be targeted for collection.

5. Active Observational Field Surveillance

Active observational surveillance (AOS) is a planned activity designed to detect evidence of disease through observation. AOS utility is highest for diseases and species that show overt clinical signs. The endpoint of AOS is recognition of symptomatic clinical signs, rather than the specific detection of VHSV. Positive AOS findings initiate follow-up diagnostic testing to investigate VHSV status.

Pilot AOS systems will be deployed in select watersheds and fish culture facilities for field testing and validation. Side-by-side comparison to probability-based diagnostic testing surveillance will facilitate field validation of AOS as a VHSV-surveillance screening tool. AOS validation studies (presuming a limited number of VHSV positive farms) could evaluate the efficacy of AOS in the general detection of clinical signs, rather than the

[8] The odds form of the Bayesian model states, posterior odds = prior odds x likelihood ratios. In this case, the posterior odds represent the probability of zone infection, prior odds represent surveillance-derived estimates of prevalence and likelihood ratios represent contextual risk.

[9] Probability = odds / (1+odds). Odds = probability / (1-probability).

[10] Surveillance estimates of prevalence will be represented by the upper 95% confidence limit of the beta distribution, using either a uniform or historical prior, describing prevalence for a set of negative surveillance findings.

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specific detection of VHSV (A. Cameron, AusVet Animal Health Services, personal communication). A validated AOS could be implemented on a broader scale to replace, or supplement, more costly (and potentially less sensitive) cross-sectional methods employed in test-based field surveillance.

Pilot systems should meet the following criteria for AOS:

- A veterinary-client relationship is established for oversight of the AOS system;
- Observers are professionally trained or receive a substantive portion of compensation for management and care of the population;
- Observers are specifically tasked with monitoring for evidence of disease, toxicity, or other causes of mortality and decreased production;
- Observations are ongoing and follow pre-planned schedules and protocols;
- The screening "test" is the observation of clinical signs. Results are used as a trigger for further investigation. The confirmatory test is laboratory testing via cell culture and confirmatory RT-PCR. Site inspection and laboratory testing of AOS positives will proceed as described for active diagnostic test-based surveillance;
- Negative and positive observations are recorded, and criteria are established for a response following positive outcomes;
- AOS practices are standardized across the industry sector under consideration.

6. Event Investigations (Appendix 7) and Movement Testing

Negative results from event investigations can be used as field surveillance data, and count toward disease freedom, if fish/site selection, sample handling and laboratory testing protocols described in this surveillance plan are followed. Mortality events are one of the preferred criteria for site selection, so should be considered a priority for this surveillance plan. States/Provinces wishing to focus all sampling efforts on mortality event investigations, assuming investigation efforts are geographically distributed across watersheds following guidelines in Table 1.1 (e.g., rather than repeatedly sampling an interesting region or event), would be supported by this plan. Sampling from mortality event investigations could also reduce the required sample size per event to 35 moribund fish (if readily available) of demonstrated VHSV susceptibility (i.e., Tier 1 or 2 species, Appendix 3). Similarly, data arising from testing prior to fish movements or translocations can be used as surveillance data if the sampling structure (and timing) is consistent with plan requirements and if the site was included in the original random site selection. Likewise, positive findings from event investigations or movement testing, if collected by approved samplers and conducted or confirmed in a Federally- or State-approved lab, will be accepted as evidence of VHSV-infection in that zone.

C. Sampling Strategy and Assumptions

Surveillance field sampling methods are targeted toward high-susceptibility environments and fish (Figure 2 and Appendix 3). Targeting may improve surveillance system sensitivity by 1) focusing resources on populations with an apparent elevated risk of VHSV IVb exposure, and 2) focusing on moribund fish which, in an outbreak situation, may have higher virus titers (and thus better virus isolation sensitivity) and disease prevalence than healthy or convalescent strata in the same population. For site selection purposes, this will include watersheds with a recent history of un-attributed mortality events, those receiving VHSV IVb susceptible species from other

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zones (either via natural or anthropogenic movements), or those with greatest intensity of recreational or commercial use or traffic. Collection sites within these subunits will be selected based on available technology and knowledge of natural or enhanced congregations of susceptible species and/or locations furthest down the drainage system (thereby exposed to upstream waters or fish movements). For fish selection purposes, targeting will focus on moribund individuals from species of known susceptibility to VHSV IVb (excluding threatened or endangered populations). Fish collection efforts should target rising or falling water temperatures in the spring and fall, respectively (concentrating efforts on temperatures under 20°C and over 2°C). Active observational surveillance for clinical and/or mortality events would help to standardize this targeting process.

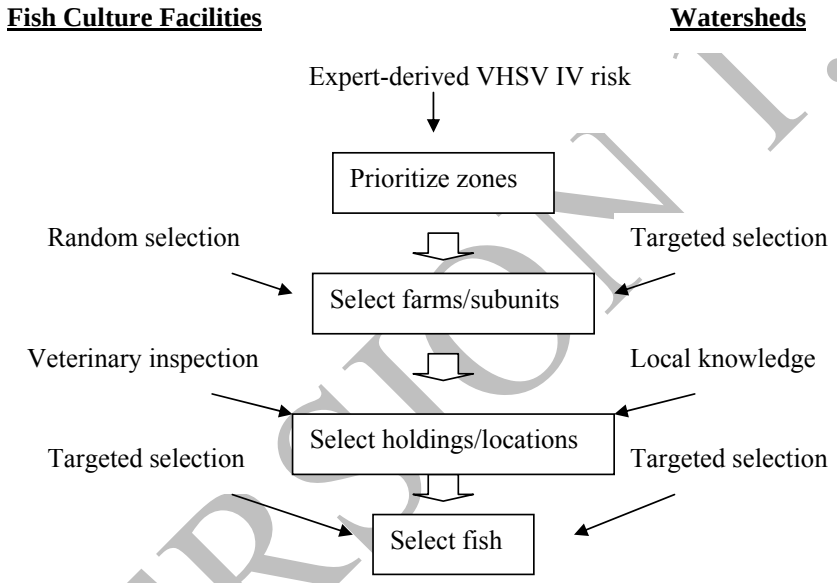

Figure 2: Schematic showing selection strategy for each stage of sampling for VHSV IVb surveillance of farmed and wild populations of freshwater fish.

Sample size calculations for disease freedom investigations involve several assumptions. The chosen diagnostic test protocol was assumed 85 percent sensitive and 100 percent specific at the level of the fish, and 95 percent sensitive and 100 percent specific at the cluster level (e.g., farm or spatial subunit). Virus isolation followed by RT-PCR for confirmation has not yet been field validated for VHSV. However, since both tests need to be positive for confirmation, specificity is likely very high. We assume 100 percent specificity because, in addition to confirmation by RT-PCR, initial zone or facility positives require field (gross- or histopathology, clinical signs or a second positive fish) and reference laboratory correspondence to confirm infection (see Case Definitions section). The estimate of test sensitivity, however, may require adjustment as more information becomes available. A pilot evaluation of test accuracy (Carol McClure, Atlantic Veterinary College, PEI, Canada, personal communication) for Infectious Hematopoietic Necrosis Virus (IHNV), a virus in the same family as VHSV, supports the importance of targeted

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sampling. The IHNV study indicates that test sensitivity improves with stage of disease progression; therefore purposefully sampling those fish with active disease should improve the sensitivity of the test. For example, in a clinical situation, sensitivity for IHNV isolation was 80 percent and specificity was 100 percent (compared to PCR). In contrast, the sensitivity for virus isolation was estimated at 35 percent with 100 percent specificity in situations where fish had recovered (sampling of healthy but still likely to be infected). Estimates for VHSV IVb sensitivity and specificity will be updated as validation results become available.

Design prevalence assumptions were set separately for each of 2 stages of sampling: first for the cluster level (e.g., facilities or subunits within a zone), and then for the fish level (fish within a facility or assemblage). For the first stage of sampling, the detection threshold for proportion of infected fish culture facilities within a zone was set at 2 percent, following OIE guidelines. However, we assumed that VHSV IVb would spread more rapidly (e.g., with fish, boats or water) among geographic subunits of a natural watershed (than among independently-operated fish culture facilities), so accepted a 10 percent threshold for detection of infected geographic subunits within a zone. Targeted selection of subunits to higher risk or higher susceptibility regions further justifies the elevated design prevalence for natural populations.

Assumptions regarding design prevalence for the second stage (fish within a population) of sampling were based on the following information. OIE guidelines on design prevalence for disease freedom investigations in fish culture populations default to 1-5 percent for slowly transmitted diseases, and > 5 percent for highly contagious diseases. Prevalence of VHSV (in wild populations) has been estimated at 4-8 percent or more in endemic situations, and much higher (e.g., greater than 50 percent) in clinical outbreaks (Hedrick et al., 2003; Skall et al., 2005). These studies, along with OIE recommendations, support a baseline design prevalence of 5% for surveillance of populations of unknown disease status. These initial studies suggest a relative risk of 10 or more for morbid vs. randomly selected fish. However, because there are few robust field prevalence studies, we selected a more conservative value for relative risk (RR = 2), and assigned twice the sampling value to fish exhibiting signs of morbidity. Relative risk defines the degree to which higher risk strata will be credited for their presumed increased disease prevalence relative to less susceptible strata. A relative risk of 2 implies that moribunds carry twice the sampling value of a fish selected at random from the general population. To keep matters simple, we'll stretch that to mean that a single moribund is worth 2 apparently healthy fish in sampling efforts to detect VHS virus.

The ability to detect diseased fish at a design prevalence of 5 percent among cultured populations, assuming 95 percent confidence, 85 percent sensitivity and 100 percent specificity, then requires sample sizes of 70 apparently healthy (representative of the general population)[11], or 35 moribund, fish per susceptible species and year class. Veterinary inspection will direct sampling efforts across holdings. All containments holding susceptible species should be examined. In the absence of veterinary concern, sampling efforts should be distributed evenly

[11] True random sampling (e.g., selection from a list of all possible fish within a group, such as species and year-class) is not attainable in most fish populations. If targeted selection of moribunds does not meet the designated sample size, the catch should be sampled systematically to achieve the balance. An example would be to select the first 70 fish collected, or every 3rd fish collected, etc., without consideration of non-targeted physical characteristics, such as length or weight.

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across the applicable holdings. However, containments with excessive mortality, clinical fish or environmental risks (stress events, final water drainage, mixed lots, etc) should be prioritized, and sampled more heavily, for selection purposes.

A slightly different set of assumptions was necessary for wild fish investigations. We presumed that a disproportionate loss of diseased fish from the general population (e.g., shortened survival of moribunds due to predation, failure to school, harsh environmental conditions, etc) and/or capture methodologies biased toward certain species or age-classes could potentially lower the prevalence of disease, and necessitate greater sampling effort, in the harvested subset of the population. Similar relative risk assumptions lead to the parallel need for greater sampling intensity for fish from mixed species/year-class lots (e.g., baitfish routinely harvested from more than one location or occasion, or forage fish raised alongside sport fish as a source of feed). Specific relative risk studies are not available for wild fish sampling for VHSV. Consequently, we based sample size calculations, for random, or systematic[10], sampling from these less controlled populations on a detection threshold of 2 percent (similar to a relative risk of 0.4), which results in sample requirements of 170 per wild, or mixed, lot or assemblage. This is similar to OIE guidelines for sampling of 150 fish from wild or mixed populations (presumably based on a 2 percent design prevalence), increased slightly to account for imperfect test sensitivity. However, in an outbreak situation, if moribund fish of susceptible species (Appendix 3) are readily obtained, specifically targeting those moribunds for surveillance purposes can reduce the necessary sample size (as described above) to 35 moribunds per assemblage.

Using a Bayesian model of infection probability, demonstration of disease freedom at these detection thresholds can be achieved either by negative (current and historical) results on surveillance, negligible risk per expert-derived risk factors, or a combination of these two evidence streams.

IV. Initial Surveillance of Wild Fish Populations

This section provides guidelines specific to surveillance of wild fish populations in watersheds. In Canada, public fish culture facilities are included in wild fish surveillance. In the U.S., public fish culture facilities are included in cultured fish surveillance.

A. Target Population

The target population includes all secondary watersheds in Canada and all 4-digit HUCs in the continental United States. These watersheds will be further subdivided to tertiary or 8-digit HUC (or smaller) subunits for sampling purposes. Table 3a shows the number of secondary and tertiary watersheds in each Province and territory in Canada. Table 3b shows the number of 4-digit and 8-digit HUCs in each State in the U.S. The total number of secondary and tertiary watersheds in Canada is 160 and 953, respectively. There is some overlap of watersheds between Provinces and the territories. The total number of 4-digit and 8-digit HUCs in the United States is 222 and 2262, respectively.

Table 3a. Secondary and tertiary watersheds in Canadian Provinces and Territories.

Province or Territory	Number of Secondary	Number of Tertiary

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	Watersheds	Watersheds
British Columbia	24	128
Alberta	27	122
Saskatchewan	24	105
Manitoba	20	103
Ontario	29	152
Québec	31	152
New Brunswick	3	37
Nova Scotia	3	45
Prince Edward Island	1	5
Newfoundland and Labrador	10	49
Yukon	11	41
Northwest Territories	28	81
Nunavut	20	99

Table 3b. HUC-4 and HUC-8 watersheds in US States. HUCs that cross State boundaries are counted in both States. Proposed surveillance would occur in a risk-adjusted subset of HUC-8s in each of the HUC-4 watersheds.

State	Number of HUC-4 Watersheds	Number of HUC-8 Watersheds
Alabama	7	53
Alaska	6	18
Arizona	10	84
Arkansas	9	58
California	16	149
Colorado	17	93
Connecticut	4	12
Delaware	2	9
District of Columbia	1	2
Florida	8	53
Georgia	9	52
Hawaii	9	9
Idaho	6	84
Illinois	11	52
Indiana	9	39
Iowa	10	56
Kansas	13	90
Kentucky	8	41
Louisiana	11	59
Maine	6	21
Maryland	5	20
Massachusetts	6	20
Michigan	14	64
Minnesota	13	81
Mississippi	8	47
Missouri	14	71
Montana	13	100
Nebraska	13	68
Nevada	12	72
New Hampshire	4	12
New Jersey	3	13
New Mexico	18	83
New York	11	53
North Carolina	9	54
North Dakota	7	50
Ohio	9	44
Oklahoma	10	67
Oregon	10	91

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Pennsylvania	10	56
Rhode Island	2	5
South Carolina	3	34
South Dakota	9	55
Tennessee	9	57
Texas	24	205
Utah	12	68
Vermont	4	16
Virginia	8	48
Washington	8	71
West Virginia	7	32
Wisconsin	12	52
Wyoming	14	83

B. Sampling Methods

1. Prioritize watersheds for surveillance

All secondary and 4-digit HUC watersheds in the United States and Canada should receive enough surveillance attention to generate a comprehensive picture of VHSV IVb risk. The amount of surveillance attention necessary to achieve that goal, however, will vary with a watershed's biological and physical characteristics and its prior history of fish health testing. Initial classification of a watershed's infection-status will be based on expert opinion-derived scoring of historical and risk factor data. The risk of VHSV IVb infection may be considered negligible for some watersheds based on risk and historical information alone. If accepted by Competent Authorities, this status could preclude the need for field surveillance. Otherwise, target sample sizes for subunits within watersheds will be adjusted by presumed risk as shown in Table 1.1.

An expert panel will establish the specific criteria and weights for the initial risk evaluation (results expected July 2007). However, just as an example, all landlocked watersheds in Canada, and all watersheds in Canada that drain into the Arctic Ocean or Hudson Bay could potentially be considered 'negligible-risk' based on historical evidence and presumed absence of susceptible species. In contrast, using these same criteria, watersheds that drain into the Pacific Ocean, including the Columbia River system, and the Atlantic Ocean would, using these criteria, be classified as 'unclassified'. Within the 'unclassified' watersheds, Lakes St. Clair, Erie, Huron and Ontario and their drainage river, the St. Lawrence River, (up to the Moose Saunders Dam and the corresponding American dam near Cornwall, ON) are already 'VHSV IVb-infected'.

In Canada, infected watersheds will not be sampled except to acquire samples for the validation study for virus isolation and qRT-PCR, to check species with unknown VHSV-susceptibility that undergo large-scale or regular human-induced movements or translocations, or to investigate fish kills and disease outbreaks. In the United States, infected watersheds will not require formal surveillance of wild fish populations, but will receive priority status for surveillance of regional fish culture facilities.

2. Construct sampling frames and target sample size

Watersheds will be divided into geographic subunits (based on existing 8-digit HUC or tertiary classification system) for sampling purposes. Where necessary to produce sufficient numbers of subunits for sampling, further subdivisions can be created using grid systems determined by the

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State or Province. The sample size estimates in Table 1.1 are derived for sampling frames with approximately 100 subunits. The number of sample sites necessary for adequate representation of a given watershed will vary by the extent of prior knowledge of VHSV risk. If historical and risk factor evidence is strong, a watershed's claim of negligible risk may be achieved with little need for test-based field surveillance. Because disease is likely to spread fairly rapidly through a watershed connected by fish, water and/or human/wildlife visitors, and because sample site selection will be targeted to high-risk locations, the selected detection prevalence for sample size calculations is 10 percent across watershed subunits. Risk-adjusted sample sizes recommended for watershed surveillance are presented in Table 1.1.

3. Target subunits for surveillance

In a given watershed, a sample of subunits will be targeted for field surveillance. Targeted selection of high-risk subunits (Appendix 3) within the watersheds should improve surveillance system sensitivity (Martin et al., 2007). Targeting criteria for selection of watershed subunits for surveillance include, in this order, current mortality events, a recent history of un-attributed mortality events, history of imports of VHSV-susceptible fish from other zones (either via natural or anthropogenic movements) and intensity of use (e.g., commercial, public or recreational rearing, harvests, boating or fishing use). In the absence of any of these criteria, watershed subunits should be selected at random, or by targeting locations of final drainage into the next watershed zone. Though the specific amount of statistical leverage achieved through these targeting criteria is unknown at this point, we presume that such sampling would at least meet, and likely exceed, the detection ability of random-based sampling. The targeting criteria used to select each subunit for surveillance purposes should be recorded.

4. Choose capture location and methodology

In each selected subunit, or subunit cluster, local knowledge will determine the most suitable location and collection methods available. For example, the collection effort within a selected site might be located either (1) where large volumes of fish from 'infected' lakes are known to have entered the waters, (2) where VHSV-susceptible fish populations are known to congregate, spawn or be most abundant, or (3) in the last lake or river that drains out of that unit. This level of sampling detail will not be prescribed, except to request that the decision criteria are recorded.

5. Target fish for collection

During the spring and/or fall, in each of 2 years, a total of 170 fish will be collected from each selected watershed subunit. Fish should be targeted (Appendix 3) by susceptible species and, if possible, by moribund appearance. Moribund fish from any susceptible species (Tiers 1 and 2) should be collected preferentially. The balance of the sample would comprise apparently healthy fish selected systematically from the highest Tier groups available. The fish assemblage (all susceptible species) present on-site during a collection event will be considered the population about which inferences can be made. Therefore, it will not be necessary to collect 170 fish from every susceptible species, but rather 170 from the assemblage. If harvest methodologies focus on a particular susceptible species, selection of 170 fish from a single (Tier 1) species is acceptable. If the surveillance visit is part of an outbreak investigation, and moribund fish of any susceptible species are readily available, 35 moribund (rather than 170 general) fish from each assemblage (or event) will suffice.

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6. Calculate probability of watershed infection

The probability of watershed VHSV infection can be calculated (by Bayesian model) from resultant field surveillance, expert opinion, and historical data. The calculated estimate can be used to inform local or Federal decisions or regulations, or it can be used to substantiate a claim of watershed disease freedom. The probability of VHSV (termed posterior probability in the Bayesian model) can be generated as a discrete number or as a continuous distribution (e.g., using WinBUGS or @Risk software). The difference is that confidence (or credibility) intervals are not tracked in the discrete form of calculation. The discrete model, however, outputs a discrete measure (rather than a distribution) of infection probability for each surveillance watershed that is easy to compare across regions. Furthermore, the discrete version is simply the product of a single value for surveillance data and a single value for risk, and is therefore transparent, intuitive and more practical for field applications. Initially, both continuous and discrete models will be developed to compare predictions and evaluate the suitability of the more user-friendly version. The final model will be automated for the end-user.

The proposed metric facilitates surveillance sampling and interpretation in three ways.

1. It provides a mechanism for the prioritization of watersheds requiring surveillance sampling and funds. Initial VHSV-risk, calculated using risk factor data, can be weighted by acreage of water suitable for farming/fishing/enhancement activities to estimate funding needs at the State/Provincial level.
2. It standardizes survey results across varying sampling intensities.
3. It provides a consistent mechanism for the incorporation of multiple knowledge streams (expert opinion, historical and current surveillance, movement testing, and disease outbreak investigation data) to estimate, and annually update, watershed VHSV IVb infection probability.

C. Specific Assumptions

- Static categorization of disease freedom is difficult to justify for an open system like a watershed. Relatively uncontrolled movements of water, fish, boats, and human/wildlife visitors suggest the need for continuous surveillance to maintain any disease freedom claims. Combining multiple streams of evidence to estimate a watershed's probability (on a scale of 0-1) of VHSV IVb infection provides an alternative approach to evaluation of disease status. Low probabilities of disease can result from a minimal presence of risk factors combined with historical negative test results and/or from current negative surveillance results. However, a watershed could still presumably achieve disease freedom status at, for example, 10 percent detection threshold, if testing of watershed units was intensive and/or the presence of known risk factors was negligible (and Competent Authorities agree to the use of non-survey data). Maintaining that status would require assurance of zonal biosecurity and/or ongoing surveillance.

V. Initial Surveillance of Fish Culture Facilities and Compartments

This section presents guidelines specific to surveillance of fish culture facilities and compartments. Surveillance zones for site selection are defined by geopolitical boundaries for the United States. In Canada, surveillance of fish culture facilities and compartments will either

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occur on a volunteer basis (commercial fish culture facilities or compartments) or will be included on the list of sampling sites in a tertiary watershed (public fish culture facilities or compartments). The rest of this section applies to surveillance of fish culture facilities and compartments in the U.S. only.

A. Target Population

In the United States, the target population is all freshwater fish culture facilities and compartments that hold and move VHSV-susceptible species.

Freshwater fish industries include public and private hatcheries to support stock enhancement and population restoration activities, and farms to hold, raise and/or propagate baitfish, sportfish, feeder fish and fish raised for human consumption. Farmed freshwater fish species cover trophic levels from forage (e.g., shiners and minnows) to predators (e.g, salmonids and muskellunge), and include a range of production facilities from indoor or outdoor tanks, to ponds or raceways, to net pens in open water. Water may be supplied via groundwater or surface water, and may or may not be treated prior to use or discharge. If, in the future, ornamental species appear on the VHSV-susceptible list, associated facilities would be included in the VHSV surveillance target population.

B. Sampling Methods

1. Prioritize zones for surveillance

States will be prioritized for VSHV IVb surveillance by the highest-risk category designated to watersheds contained within, traversing or abutting State boundaries. Risk categorization and sample size adjustments for disease freedom investigations will proceed as described for watersheds. However, for groupings of fish culture facilities, in contrast to groupings of watershed subunits, the disease detection threshold will be set at 2 percent (OIE manual).

2. Random Site Selection

A list will be obtained for each zone (e.g., State) of all freshwater fish culture facilities currently or historically involved in live fish sales, exchange or stock enhancement of known-susceptible species with any other zones, and either willing or State-mandated to participate in surveillance. From this registry, a subset of facilities will be selected at random to test the hypothesis of disease freedom at a design prevalence of 2 percent with 95 percent confidence. A random selection of facilities will be generated, and visited/sampled as described, twice a year for 2 years to assess disease status.

3. Veterinary Inspection

An APHIS-accredited veterinarian will visit each selected site to perform a clinical inspection of all holdings of VHSV IVb susceptible species. Tanks, ponds, raceways or cages thought most vulnerable to disease (for example, by final distribution of shared water, mixed age-class or species lots, wild harvest origin, young or reproductive fish, recent stressors, or recent un-attributed mortality or clinical disease) will receive preferential focus.

4. Targeted Fish Selection

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Fish will be grouped by species and age-class (rather than by containment) for surveillance sampling purposes. Following veterinary inspection, a representative selection of fish will be selected for testing from each species/age-class grouping. Whenever possible, sampling should target moribund fish. For species managed as single year-classes, 35 moribund or 70 healthy fish will be selected from each. If moribunds are available, but total less than 35, the balance would be achieved through systematic selection of apparently healthy counterparts. In the case of a mixed moribund/healthy sample, moribunds would equal two healthy fish in surveillance value, bringing the total value of fish sampled to 70. The equation would read: (2 x moribund submissions) + (1 x healthy submissions) = 70. These numbers assume population sizes exceeding 500 fish, 95 percent confidence, 85 percent sensitivity and 100 percent specificity. The design prevalence for detection of disease is set at 5 percent across the population of fish, with relative risk assumed highest for moribund strata. This decision is supported with previous estimates of VHSV prevalence around 5% or higher in endemic situations (Hedrick et al., 2003; Skall et al., 2005) and exceeding 50% in natural outbreaks (Hedrick et al., 2003). In addition, OIE recommendations default to over 5% for relatively contagious pathogens. FreeCalc can be used to determine sample sizes required for smaller populations. For species managed as mixed-lots, including baitfish routinely harvested from more than one location and occasion, a fixed total of 170 fish will be sampled from each mixed lot (following OIE guidelines, and reduced relative risk assumptions for the observable fraction of mixed or wild lots).

5. Evaluate Disease Freedom

Negative results from this surveillance would be sufficient to claim VHSV freedom (95 percent confidence), at a 2 percent detection threshold for registered fish culture facilities within the zone (and a 5 percent detection threshold for fish within a facility). If associated watersheds are also considered low risk, the zone itself can be claimed VHSV-free.

6. Specific Assumptions

- Two percent design prevalence (95 percent confidence) at the fish culture facility or compartment level is low enough to resume or maintain trading partner confidence for the zone. This level was based on OIE guidelines for surveillance of aquatic animal diseases.
- Administrative animal health regulations and oversight effectively minimize the risk of virus introduction into the zone and/or surveillance for VHSV is ongoing.

VI. Ongoing Surveillance of Fish in Fish Culture Facilities, Compartments or Wild Fish Populations

The minimum requirements for continued surveillance that result from any designated risk status are depicted in Figure 2 (and Table 2). Disease-free zones that can demonstrate functional biosecurity and disease-reporting capabilities need only annually revise their (Bayesian model) VHSV IVb infection probability for associated watersheds. Adoption of AOS in this setting would provide ongoing reassurance of disease freedom. Disease-free zones that cannot demonstrate functional biosecurity, and zones that have not yet established disease freedom, should continue field surveillance with an approved testing regimen (either a validated AOS or ongoing surveillance) and annual revision of their (Bayesian model) probability of VHSV IVb infection. Infected zones that achieve eradication should conduct ongoing surveillance to begin

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to accumulate evidence of disease freedom. Alternatively, infected zones that have not or can not achieve eradication should consider compartmentalization as an option to negotiate trade from distinct segments within the zone.

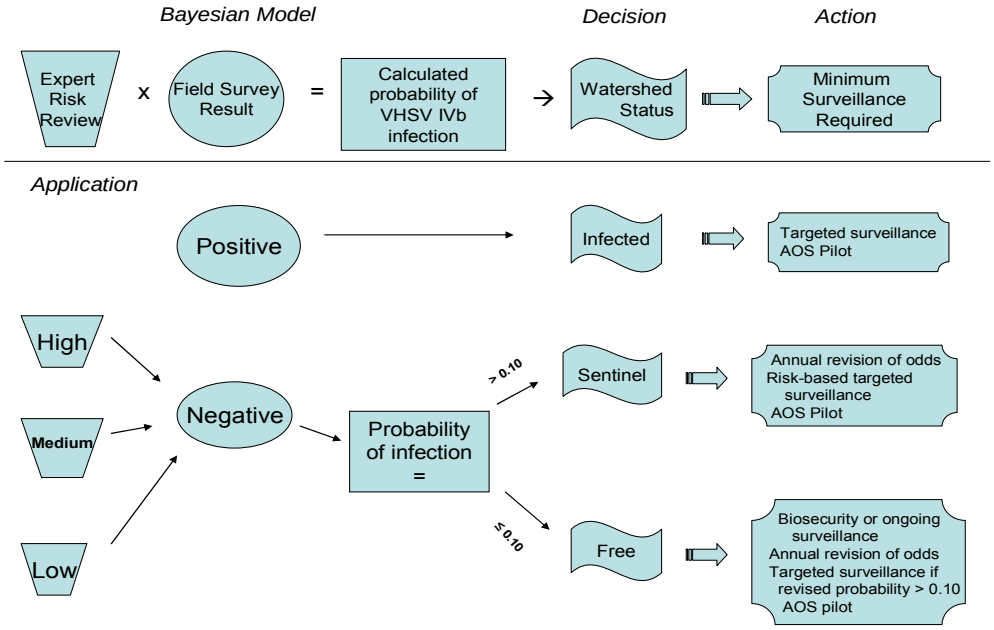

Figure 2. Requirements for watershed surveillance based on calculated VHSV-risk status.

VII. Data Capture, Reporting and Presentation

A. Data Entry

There will be one row of data for each individual fish that is sampled. Data entry will be conducted by Federally-designated individuals. Required data fields for each row will include (among others): fish reference number, laboratory reference number, Federal reference number, State/Province reference number, wild or cultured origin, watershed zone ID, State/Province ID, sampling site location (at least two data fields consisting of State/Province, GIS coordinates of site, and/or common name of site), sampling date, sampling time, water temperature of holding or collection site, targeting criteria used to select the site or holding, targeting criteria used to select the fish, fish species scientific name, fish species common name, year-class (if cultured), type of samples, name of dissection/sample processing laboratory, results of cell culture, results of RT-PCR, results of genotyping, and results of AOS. Data fields associated with the watershed (or facility) will include results of historical data, results of expert opinion survey, and results of demographic survey.

B. Database Management

Each country (at the Federal level) will design and manage an independent database. However, the following components must be standardized between countries and agencies: database software, database design, database fields and database coding of variables. Data sharing details within/between countries/agencies will be provided when available.

C. Data Use and Presentation

Data use will occur at several levels: local level for management decisions; State/Provincial and Federal level for regulatory purposes; Federal level for performance evaluation and validation.

VIII. Surveillance system implementation and evaluation

A. Implementation Priorities

Outreach education, database development, and field sampling and laboratory coordination are critical first steps in the implementation of VHSV surveillance. As further funds become available, field surveillance assistance would be provided.

B. Resources: Allocation of Surveillance Funds (U.S. only)

Allocation of surveillance funds to States could be based on VHSV-risk to their entire jurisdiction. Surveillance goals support an allocation scheme that accounts for State (1) acreage suitable for fish culture, enhancement/conservation or fisheries activities, adjusted by (2) the relative pre-surveillance risk of VHSV infection, as predicted by risk factors and historical data, in constituent watersheds.

C. VHSV-Surveillance Review and Performance Metrics

An evaluation of the initial VHSV IVb surveillance will be conducted by the Bilateral VHSV Surveillance Committee in order to provide recommendations on surveillance plan updates or modifications. This review will include stakeholder feedback, summary statistics, surveillance system sensitivity, field validation of virus isolation and RT-PCR, field validation of alternative testing modalities, pathogen distribution and prevalence evaluations where possible. The assessment will be conducted following NSU guidelines for the evaluation of animal health surveillance systems.

IX. Definition of Terms

Active Observational Surveillance (AOS): The process of actively and systematically looking for diseased animals by a knowledgeable individual, on a frequent, pre-planned, and ongoing basis, where a predefined plan of action is implemented when affected animals are discovered.

Basic Biosecurity Conditions (2006 OIE Aquatic Animal Health Code): A set of conditions applying to a particular disease, and a particular zone or country, required to ensure adequate disease security (For details, see definition for 'Compartment').

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Case Definition: Specifies the criteria that define a VHSV-positive fish, fish culture facility, watershed, zone, or compartment. See VHSV IVb Classification section (X) below for VHSV IVb specific case definitions.

Commercial Fish Culture Facility: A fish culture facility that is privately owned and operates as a business.

Compartment (2006 OIE Aquatic Animal Health Code): One or more fish culture facilities under a common biosecurity management system containing an aquatic animal population with a distinct health status with respect to a specific disease or diseases for which required surveillance and control measures are applied and basic biosecurity conditions are met for the purpose of international trade. Such compartments must be clearly documented by the Competent Authority.

For the purposes of this surveillance plan, a compartment is a fish culture facility with the following characteristics:

a) Biosecurity (i.e., separation from the environment)
    a. Protected water source (ground or treated surface water if in a positive zone)
    b. Equipment, personnel and vessel cleaning/disinfection practices
b) Movement controls
c) A documented health program
d) Management practices with documented standard operating procedures, and
e) Association with a licensed veterinarian or experienced (> 1 year) fish health practitioner

For the purposes of this surveillance, sampling of compartments will occur at the containment level (e.g., tanks, ponds, raceways, etc.) and at the fish level. It is recognized for the purposes of this surveillance plan that compartmentalization can also be achieved within a compartment.

Competent Authorities: (2006 OIE Aquatic Animal Health Code). Refers to the Authorities of a Member Country that have the responsibility and competence to ensure or supervise the implementation of aquatic animal health measures or other standards in the OIE Aquatic Code.

The Competent Authority in Canada is the Canadian Food Inspection Agency (CFIA), Aquatic Animal Health Division (AAHD). The Competent Authority in the U.S. is the US Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) Veterinary Services. An MOU exists between USDA APHIS, the U.S. Fish and Wildlife Service (USFWS) and U.S. National Oceanic and Atmospheric Administration (NOAA) for the signing of health certificates for aquatic animals under their respective jurisdictions.

Local regulatory authorities include the State agencies and Provincial ministries recognized by the Competent Authority to be responsible for the supervision and implementation of aquatic animal health measures in their State or Province.

Containment: A structure, e.g., raceway, cage or pond, holding a distinct group of fish. Any given farm may have multiple individual containments.

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Cultured Fish: For the purposes of this surveillance plan, cultured fish refers to any contained fish that meets the definition (below) for either farm-raised or farm-managed.

In the U.S., surveillance for VHSV among farm-raised and farm-held populations will follow the same guidelines (under cultured fish populations). However, regulatory response will necessarily differ between farm-raised and farm-managed populations based on available biosecurity measures. Definitions for cultured fish sub-types are currently in progress in Canada.

Disease Freedom: A designation applied to zones or compartments that can demonstrate, with an accepted statistical level of confidence, a negligible likelihood of the presence of a certain disease or pathogen. See Section III for VHSV IVb specific definition of disease freedom.

Fish Culture Facility: A facility in which fish for breeding, stocking or marketing are raised or kept. For the purposes of this surveillance plan, fish culture facility includes fish farms, net cages or ponds with bait fish (if they are held long enough to require feed), enhancement facilities, and enhancement and restoration activities. See Commercial and Public Fish Culture Facilities.

In the U.S., both farm-raised and farm-managed fish populations will follow the same (cultured fish populations) surveillance protocols. However, farm-raised and farm-managed populations may fall under different regulatory and/or compensation requirements. Definitions for culture facility types are currently in progress in Canada.

Farm-managed Fish (U.S. only): Refers to contained aquatic animals that do not meet the definition for farm-raised fish, but that do experience human intervention to enhance production (such as regular stocking, feeding, protection from predators, etc.).

Farm-raised Fish (U.S. only): Refers to contained aquatic animals that are hatched and raised in a controlled environment. This definition includes enhancement activities and may include restoration activities. For the purposes of this surveillance plan, egg-takes from wild fish, where disinfection is applied, can be included in this definition.

Fish Health Inspector: In Canada, all fish sampling will be under the auspices of the CFIA. Wild fish will be sampled by Federal or Provincial employees or their designates. Fish in commercial fish culture facilities will be sampled by a licensed veterinarian or by a Federal or Provincial employee.

Fish sampled from U.S. fish culture facilities will be selected and submitted by APHIS VS Area office, APHIS-accredited or State veterinarians. Wild fish sampled from natural watersheds will be selected and submitted by State-recognized fish health authorities (e.g., APHIS-accredited veterinarian, American Fisheries Society-certified biologist, or Federal- or State- designated employee). States may propose an alternative set of professional requirements necessary for VHSV fish health investigations in that State, e.g., for State or Federal hatcheries, or if sampling needs exceed capacity. State-determined professional requirements for VHSV fish health inspectors should be clearly documented, available to other States, and accepted by the Competent Authority for fish health, and the VS aquaculture liaison, for the State. In all cases,

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sampling for confirmatory purposes of commercial farm-raised populations will require veterinary submission as described above.

Freshwater: A zone or compartment is considered freshwater if the water supply has a salinity of 0 to 5 ppt. A coastal watershed is considered freshwater down to its natural tidal limit.

Public Fish Culture Facility: A fish culture facility that is managed by Federal or State/Provincial governments, a government-public partnership, or by the public. These establishments are not businesses.

Random Sampling Simple random sampling is the selection of a subset of the population (e.g., sites or fish) where every member has an equal probability of being selected. This is typically achieved by creating an exhaustive list of all members of the population, and drawing numbered observations from that list using a random numbers generator or table.

Subunit: For the purposes of this surveillance plan, subunits refer to geographic parcels that result from the systematic division of a watershed into a series of sampling units. Subunits may result from the division of a HUC-4 or secondary watershed into its constituent HUC-8 or tertiary watershed units. Alternatively, HUC-4 or secondary watersheds may be divided into constituent subunits through other defined grid systems accepted by the Competent Authority.

Systematic Sampling: The selection of a subset of caught fish based on selection of every k^{th} unit. For the purposes of this surveillance plan, for populations of unknown size (e.g., the number of fish harvested on a particular site and date), k can be estimated as 1 for populations approximating the final sample size, and 2 or more for larger populations. The goal of systematic sampling is to minimize sampling bias (i.e., intentional or unintentional selection of non-targeted traits such as length or weight) in situations where random sampling is not possible.

Targeted Surveillance or Selection: The selection of a subset of sites or fish that is likely to exhibit a higher prevalence (relative risk) of VHSV infection if it is present. Such selection reduces the sample size requirements necessary to demonstrate disease freedom at a pre-determined design prevalence.

Watershed: Watershed is defined at the secondary level in Canada, and at the level of the 4-digit Hydrological Unit Code (HUC) in the United States. Geographic subunits for surveillance sampling of watersheds are initially defined at the level of the tertiary watershed in Canada, and the 8-digit HUC in the U.S. (see subunit definition).

Wild Fish: Fish living in natural water bodies or drainages that are not considered a part of fish culture or farm facilities as defined above. This definition includes feral fish residing in natural waters, even if a portion of their life cycle was, or is, managed in a farmed setting.

Zone (2006 OIE Aquatic Animal Health Code): A portion of one or more countries comprising: a) an entire water catchment from the source of a waterway to the estuary or lake, or b) more than on water catchment, or c) part of a water catchment from the source of a waterway to a barrier that prevents the introduction of a specific disease or diseases, or d) part of a coastal area

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with a precise geographical delimitation, or e) an estuary with a precise geographical delimitation, which consists of a contiguous hydrological system with a distinct health status with respect to a specific disease or diseases. The zones must be clearly documented (e.g., by a map or other precise locators such as GPS coordinates) by the Competent Authorities.

A VHSV surveillance zone may comprise :
1) A region described by geopolitical boundaries (e.g., State or Province), or
2) A contiguous hydrological system described by
   a) an entire water catchment from the source of a waterway to the estuary or lake, or more than one water catchment, or
   b) part of a water catchment from the source of a waterway to a barrier that prevents the introduction of a specific disease or diseases, or
   c) part of a coastal area with a precise geographical delimitation, or
   d) an estuary with a precise geographical delimitation.

X. VHSV Classification System

This section provides technical VHSV-classification definitions. Zone refers to the system of data aggregation and may be based on geopolitical (e.g., State or Province) or watershed boundaries (see definition of Surveillance Zones in Part III).

A. VHSV-Infected

- **Fish:** A fish will be considered infected with VHSV if an APHIS- or DFO-approved laboratory, using accepted protocols, reports it positive for any strain of VHSV by virus isolation on cell culture and subsequent confirmation by RT-PCR. Initial positives from a new species, facility, compartment or zone will require confirmatory identification by U.S. or Canadian Federal Reference Laboratory (NVSL in the U.S. and PBS-DFO in Canada). All individual RT-PCR confirmed VHSV isolates (or a sub-group if more than 3 positives are recovered from a single sampling event) will be sequenced for genotype identification.

- **Fish Culture Facility or Compartment:** A fish culture facility or compartment will be considered infected with VHSV if BOTH of the following conditions are met:
  1. Condition 1, laboratory evidence, is met if one (or more) fish collected from that compartment tests positive for VHSV as described above.
  2. Condition 2, field evidence of VHSV establishment, is met by one or more of the following:
     (a) a second positive fish originates from a separate species or sampling event, OR
     (b) gross-pathology (determined by fish-health inspector) or histopathology (determined by lab personnel) is consistent with VHSV in one or more test-positive fish.

  Initial positives from a facility or compartment will require confirmation by U.S. or Canadian Federal Reference Laboratory (NVSL in the U.S. and PBS-DFO in Canada).

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- **Watershed:**
  A HUC-4 or secondary watershed will be considered infected with VHSV if BOTH of the following conditions are met:
  1. Condition 1, laboratory evidence, is met if one (or more) fish collected from wild fish or cultured populations from any subunit in that watershed (unless the population is considered a compartment) tests positive for VHSV.
  2. Condition 2, field evidence of VHSV establishment, is met by one or more of the following:
     (a) a second positive fish originates from a separate species or sampling event, OR
     (b) gross-pathology (per fish health inspector) or histopathology (per lab personnel) is consistent with VHSV in at least one test-positive fish.

  Initial positives from a watershed will require confirmation by U.S. or Canadian Federal Reference Laboratory (NVSL in the U.S. and PBS-DFO in Canada).

- **Geopolitical Zone (e.g., State) (U.S. only):** A geopolitical zone will be considered infected with VHSV if BOTH of the following conditions are met:
  1. Condition 1, laboratory evidence, is met if one (or more) fish collected from fish culture facilities within the zone, or from HUC-4 watersheds adjoining, traversing or contained within the zone, tests positive for VHSV (unless the infected population is considered a compartment).
  2. Condition 2, field evidence of VHSV establishment, is met by one or more of the following:
     (a) a second infected fish originates from a separate species or sampling event, OR
     (b) gross-pathology (per fish health inspector) or histopathology (per lab personnel) is consistent with VHSV in at least one test-positive fish.

  Initial positives from a State or Province will require confirmation by U.S. or Canadian Federal Reference Laboratory (NVSL in the U.S. and PBS-DFO in Canada).

B. VSHV-Suspect Fish Population (AOS or Disease Investigations only)

- A population, or group of fish, will be considered suspect for infection with VHSV if active observational surveillance or outbreak investigations note two or more fish exhibiting the following conditions in the absence of another attributed cause:
  1. Clinical signs consistent with VHSV infection for the fish species under investigation, including two or more of the following: rapid onset of mortality, exophthalmia, hemorrhages at base of bins, gills, eyes and skin, abnormal swimming patterns such lethargy, flashing and spiraling, and distended abdomen; OR
  2. Gross pathology consistent with VHSV infection for the fish species under investigation, such as: kidney is dark red, swollen and/or necrotic, liver is pale and mottled, heart has a ground glass appearance, petechial hemorrhages in skin, muscle tissue and/or internal organs, swollen spleen, the gastrointestinal tract is devoid of food, appearance of vesicles or hemorrhage in the swim bladder; OR

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3. Histopathology consistent with VHSV infection for the fish species under investigation, such as, but not limited to: kidney, heart and/or spleen show extensive focal necrosis and degeneration (cytoplasmic vacuoles, pyknosis, karyolysis, lymphocytic invasion), and accumulation of erythrocytes in skeletal muscle bundles and fibers.

- Report of suspect populations will trigger follow-up investigations conducted in conjunction with a fish health inspector (see definition) under the supervision of an APHIS- or DFO-accredited or approved veterinarian or the region's Competent Authority for fish health. Fish will be selected for diagnostic testing as described in Section III--Fish selection methods. A suspect population will only be classified VHSV-infected if follow-up investigation by an approved fish health inspector meets the criteria for VHSV-infected status as described, in section A, above.

C. VHSV-Unclassified

- **Compartment, Watershed Zone or Geopolitical Zone:** A compartment or zone will be considered VHSV-unclassified if it can not yet be classified VHSV-infected or VHSV-free. Unclassifed status presumes BOTH of the following conditions:
    1. Expert-derived evaluation of contextual risk is considered greater than negligible (Tables 1 and 2).
    2. Surveillance and/or biosecurity measures are inadequate to declare or maintain freedom status:
    (a) Surveillance efforts have not yet established disease freedom or VHSV-infection; and/or
    (b) Biosecurity measures to prevent new introductions of disease (e.g., in regions that have demonstrated statistical disease freedom) cannot be demonstrated.

  Infection probability (on a scale from 0 to 1) for VHSV IVb susceptible watersheds will be described using a Bayesian model to combine empirical (current and historic) survey results and expert-derived assessment of risks.

D. VSHV-Free

- **Compartment:** A individual compartment will be classified VSHV-free if evaluation confirms conditions 1, 2 and 3 below:

1. Condition 1, negative baseline surveillance, is met by one or more of the following:
    (a) Absence of susceptible species.
    (b) Two years of twice-yearly (spring and fall) surveillance testing is negative across randomly-sampled containments. Specifically, surveillance should ensure 95% confidence in detecting diseased fish within a given containment at a design prevalence of 5 percent[12], and across containments within a farm at a design prevalence of 2 percent.

[12] We can achieve this 5% design prevalence at the fish-level by sampling 70 healthy or 35 moribund fish from individual containments.

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(c) Two years of twice-yearly (spring and fall) surveillance testing, as described in this surveillance plan, is negative for each susceptible species and year class on the facility. Specifically, surveillance should ensure 95% confidence in detecting disease at a design prevalence of 5 percent within each species/year-class population (or lot, if mixed class) of susceptible fish[13] on the farm. Under this approach, the proportion of samples taken from individual holdings of a particular species/year-class would be determined by veterinary inspection, and distributed either uniformly or by targeting high susceptibility containments.

2. Condition 2, adequate biosecurity, is met if biosecurity conditions (a 10-year history is required in the absence of structured testing; see definition of Compartment) and disease reporting protocols to protect the compartment from new VHSV introductions are documented and maintained.
3. Condition 3, ongoing surveillance, is met by either of the following:
   (a) Ongoing observational surveillance (passive or active) is practiced for compartments in low VHSV-risk watersheds (e.g., risk score ≤ 0.1); or
   (b) Ongoing systematic surveillance (either validated AOS or diagnostic test-based) is practiced for compartments in higher-risk (risk score > 0.1) watersheds.

- **Watershed:** Wild fish populations in a HUC-4 or secondary watershed will be classified VSHV (IVb)-free if VHSV surveillance is negative for VHSV, and if biosecurity and ongoing surveillance are addressed. Conditions 1, 2 and 3 below must all be met.
  1. Condition 1, negative wild fish surveillance, is met if one or more of the following statements is true:
     (a) Susceptible species are absent and there is no history of VHSV occurrence.
     (b) VHSV risk, per expert-derived evaluation of contextual factors, is considered negligible (Table 1).
     (c) Two years of once a year (spring or fall) testing of fish populations from selected watershed subunits, as described in this plan, is negative for confirmed VHSV. This surveillance guides sampling intensive enough to detect VHSV (95% confidence) if infection is present in ≥ 10 percent of targeted subunits[14], and ≥ 5 percent of the general population of fish[15] in an infected region.
     (d) A Bayesian model combining field surveillance and risk factor evidence streams calculates the watershed probability of VHSV infection ≤ 0.10.
  2. Condition 2, adequate biosecurity, is met if biosecurity conditions and disease reporting protocols to protect the watershed from new VHSV introductions are documented, accepted by the Competent Authorities, and maintained.

[13] We can achieve this 5% design prevalence at the fish-level by sampling 70 healthy or 35 moribund fish from individual species/year-class cohorts (with sampling effort distributed across containments per veterinary inspection). Mixed species/year-class populations require more intensive sampling (170 fish per lot or assemblage). However, a clinical outbreak of disease in a mixed population would reduce sampling requirements to 35 moribunds per population.

[14] We can achieve this 2% design prevalence at the site-level by conducting VHSV surveillance at a risk-adjusted number of targeted watershed subunits as shown in Table 1.1.

[15] We can achieve this 5% design prevalence at the wild fish-level by sampling 170 general fish from the captured population of susceptible species. However, a clinical outbreak of disease in a wild population would reduce the necessary sample size to 35 moribunds per captured assemblage.

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3. Condition 3, ongoing surveillance, is met if either of the following is true:
   (a) Watersheds (HUC-4 or secondary) comprising, crossing or abutting the geopolitical zone maintain low-risk status (risk score ≤ 0.1).
   (b) Wild fish surveillance (either structured test-based or validated AOS) is ongoing (and sufficient to detect disease at 10 percent prevalence across subunits) if the watershed is considered high-risk (risk score > 0.1).

It is recognized, for the purposes of this surveillance, that a watershed may establish disease freedom if biosecurity conditions are in place and approved by Competent Authorities and VHSV surveillance testing is negative.

- **Fish Culture Facilities within a Zone:** Registered fish culture facilities within a declared zone will be classified VSHV (IVb)-free if farmed surveillance is negative, and biosecurity and ongoing surveillance are addressed. Conditions 1, 2 and 3 must be met.
  1. Condition 1, negative cultured fish surveillance, is met if any of the following is true.
     (a) Susceptible species are absent and there is no history of VHSV occurrence.
     (b) VHSV risk for the zone, per expert-derived evaluation of contextual factors, is considered negligible (Table 2).
     (c) Two years of twice-yearly (spring and fall) testing of registered fish culture facilities, as described in this surveillance plan, is negative for confirmed VHSV. This surveillance guides sampling intensive enough to detect VHSV (with 95% confidence) if infection is present in ≥ 2 percent of registered facilities[16], and ≥ 5 percent of the general population of fish[12] in an infected facility.
  2. Condition 2, adequate biosecurity, is met if biosecurity conditions and disease-reporting protocols to protect the zone from new VHSV introductions are documented, accepted by the Competent Authorities, and maintained.
  3. Condition 3, ongoing surveillance, is met if either of the following is true:
     (a) Watersheds (HUC-4 or secondary) comprising, crossing or abutting the geopolitical zone maintain low-risk status (risk score ≤ 0.1).
     (b) Surveillance (either structured test-based or validated AOS) of registered fish culture facilities is ongoing (and sufficient to detect disease at 2 percent prevalence) if associated watersheds are considered high-risk (risk score > 0.1).

- **Geopolitical Zone (e.g., State):** A geopolitical zone will be classified VSHV (IVb)-free if both wild and farmed surveillance are negative, and biosecurity and ongoing surveillance are addressed. Conditions 1, 2, 3 and 4 must all be met.
  1. Condition 1, negative wild fish surveillance, is met if one or more of the following statements is true:
     (a) Susceptible species are absent and there is no history of VHSV occurrence.
     (b) VHSV risk, per expert-derived evaluation of contextual factors, is considered negligible (Table 1).
     (c) Two years of once a year (spring or fall) testing of fish populations from selected watershed subunits, as described in this surveillance plan, is negative for confirmed VHSV. This surveillance guides sampling intensive enough to detect

[16] We can achieve this 2% design prevalence at the site-level by conducting VHSV surveillance at a risk-adjusted number of randomly-selected facilities as shown in Table 1.2.

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VHSV (95% confidence) if present in ≥ 10 percent of targeted subunits[12], and ≥ 5 percent of the general population of fish[13] in an infected region.

2. Condition 1, negative cultured fish surveillance, is met if any of the following is true.
   (a) Susceptible species are absent and there is no history of VHSV occurrence.
   (b) VHSV risk for the zone, per expert-derived evaluation of contextual factors, is considered negligible (Table 2).
   (c) Two years of twice-yearly (spring and fall) testing of registered fish culture facilities, as described in this surveillance plan, is negative for confirmed VHSV. This surveillance guides sampling intensive enough to detect VHSV (with 95% confidence) if infection is present in ≥ 2 percent of registered facilities[15], and ≥ 5 percent of the general population of fish[12] in an infected facility.
3. Condition 3, adequate biosecurity, is met if biosecurity conditions and disease-reporting protocols to protect the zone from new VHSV introductions are documented, accepted by the Competent Authorities, and maintained.
4. Condition 4, ongoing surveillance, is met if either of the following is true:
   (a) Watersheds (HUC-4 or secondary) comprising, crossing or abutting the geopolitical zone maintain low-risk status (e.g., risk score ≤ 0.1).
   (b) Surveillance (either structured test-based or validated AOS) of high-risk watersheds (risk score > 0.1), and associated fish culture facilities, is ongoing and sufficient to detect disease at 10 percent (among watershed subunits) and 2 percent (among registered fish facilities) design prevalence respectively.

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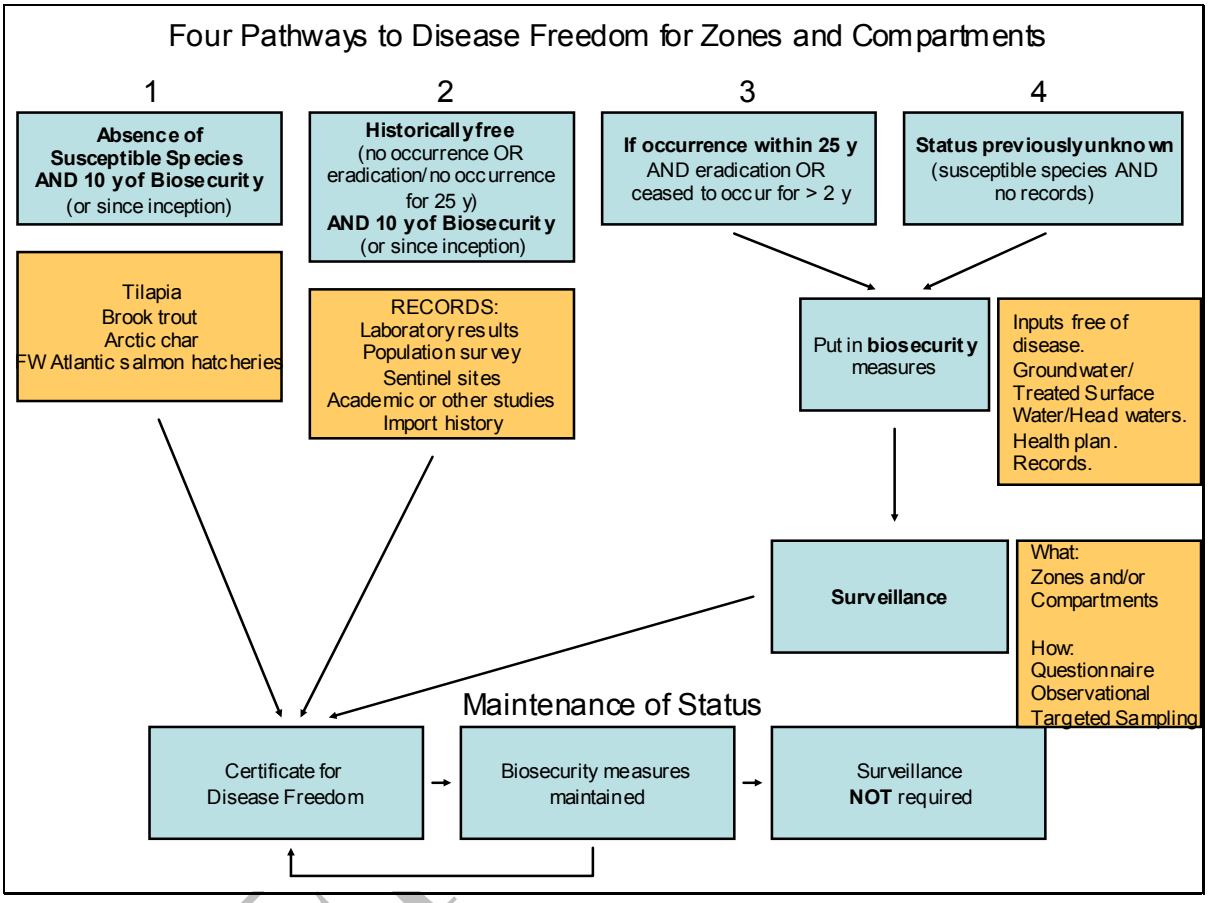
Appendix 1: OIE Guidelines for Disease Freedom



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Appendix 2: Maps of Watershed Zones in the U.S. and Canada

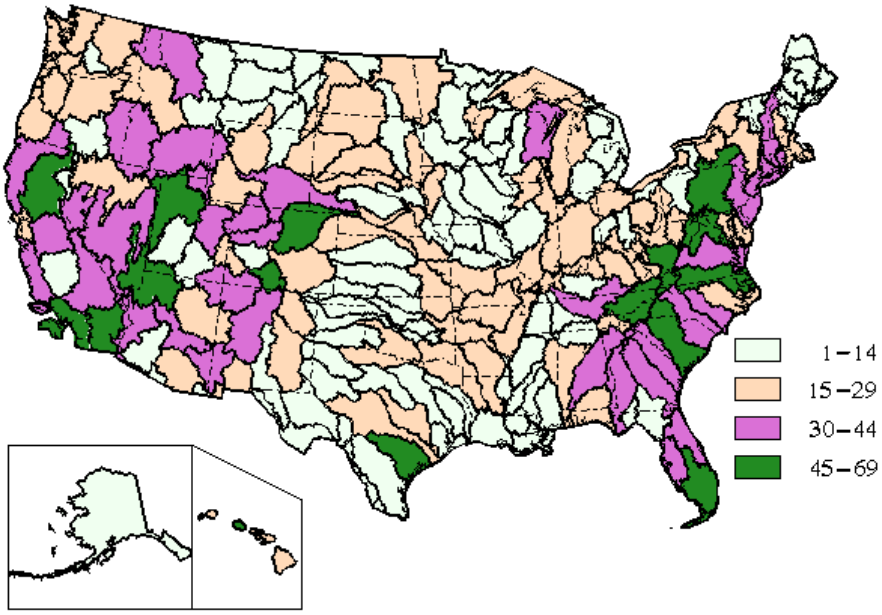

Figure 1 Map of the 4-digit HUC division of the United States of America, obtained from the USGS Web site, available at http://nas.er.usgs.gov/hucs.asp, accessed April 4, 2007. The color-coding is not relevant to the VHSV surveillance plan

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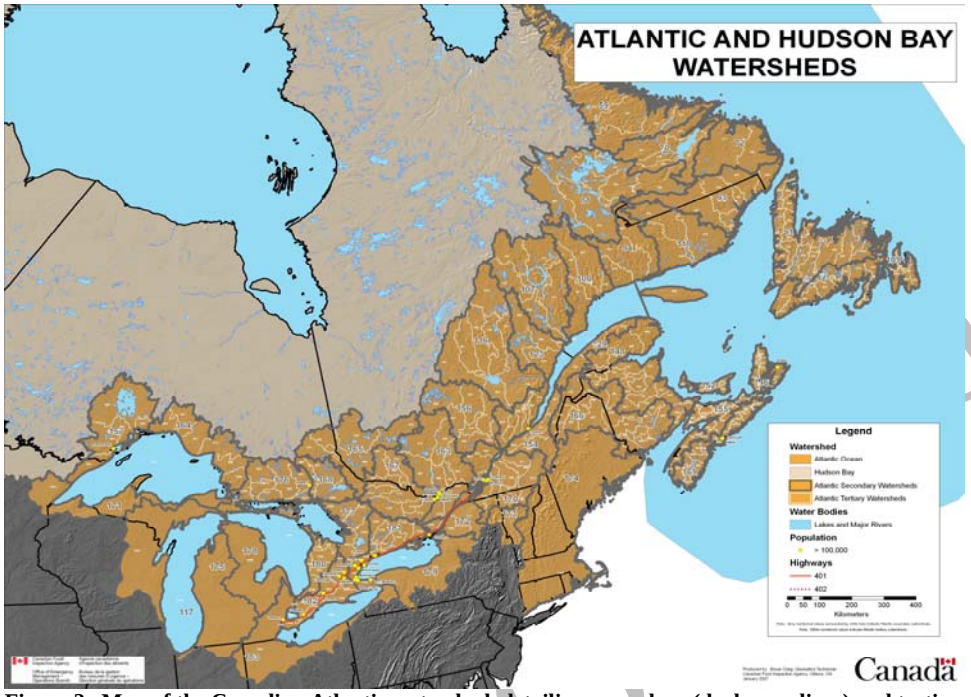

Figure 2 Map of the Canadian Atlantic watershed, detailing secondary (dark grey lines) and tertiary (light grey lines) watersheds.

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Appendix 3: Targeted Surveillance for Selection of Sites and Fish

Targeted surveillance may be used in surveillance plans that do not require an estimation of prevalence. Since we are only interested in absence or presence of the pathogen, surveillance can be targeted to those watersheds and fish that are likely to harbor the virus and living under appropriate environmental conditions based on current knowledge of the disease in the United States and Canada. These criteria can be used to more effectively target sampling for VHSV; more effective sampling decreases the number of sites and fish that need to be sampled.

Targeting Criteria

Several known factors enhance the detection of VHSV using laboratory testing procedures. These factors are listed in Table 4 below. Detection of VHSV is easier when the prevalence of infection is high; that is, when more fish are infected ('prevalence' is a term that describes the number of fish in the group that are infected). The ability of laboratory testing to find the virus improves when each fish carries a lot of virus, as the sample requires a minimum number of virus particles for the test to detect them.

The table below (Table 1) shows criteria that contribute to increased prevalence of infection in a population of fish (more fish are infected in the group), and criteria that contribute to more virus per fish (increased within fish prevalence because of increased viral load per fish).

Table 1. Criteria that classify fish and sites into higher VHSV prevalence strata, assuming that VHSV is present. This table will be updated as more information becomes known.

Criterion	Increase Fish Population Prevalence	Increase Within Fish Prevalence
Susceptible Fish Species	Yes – see Tier Sampling of Fish Species in Table 2 below	Yes – if fish are young, sexually mature or diseased
Age of Fish	Yes – young fish	Yes – if young or sexually mature
Life Stage of Fish	Yes – early post-hatch, reproducing adults	Yes – if early post-hatch, sexually mature or diseased
Sick Fish	Yes – if VHSV is a cause of illness	Yes – if VHSV is a cause of illness
Fresh Dead Fish	Yes – if VHSV is a cause of death	Yes – if VHSV is a cause of illness
Water temperature at Site	Yes – < 20˚C	Yes – 4˚C to 18˚C
Season at Site	Yes – Spring, when water temperatures are rising and/or Fall, when water temperatures are falling	Yes – 4˚C to 18˚C
Site receives Fish	Yes – if large volumes are received and the fish are susceptible	Yes – if fish are young, sexually mature or diseased

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Criterion	Increase Fish Population Prevalence	Increase Within Fish Prevalence
Site experiences Migratory Fish Populations	Yes – if migratory fish are susceptible	Yes – if fish are young, sexually mature or diseased

Three methods of surveillance take advantage of the criteria:

1. Observational (disease detection) surveillance: looking for sick fish with general syndromic signs of septicemia. This is most easily done in culture facilities or wherever groups of fish are captured for any purpose. All sick fish, or a pre-determined number, are sampled for laboratory testing.
2. Investigation of mortality events (fish kills or disease outbreak investigations): sampling fresh-dead or moribund fish for detection of infection with VHSV. The probability of detecting the virus is increased if fish exhibit general syndromic signs of septicemia. A pre-determined number of dead fish are sampled for laboratory testing.
3. Targeted surveillance of populations of fish that are healthy (illness and mortality events are below expected levels) where sites and fish are selected for sampling because they are expected to have a higher prevalence of VHSV infection if it is present.

The first two surveillance methods take advantage of criteria that increase both the fish population prevalence and the within fish prevalence: sick fish and dead fish. The least number of samples are required for these methods.

The third surveillance method samples healthy fish so a combination of sampling criteria (susceptible fish species, age, life stage, and water temperature) are required to enhance fish population prevalence and within fish prevalence in order to reduce the overall number of fish to be sampled. No combination of these criteria can reliably predict the prevalence of VHSV among and within fish, except when there is opportunity to include sick or fresh-dead fish in the sample, but selection based on multiple criteria increases the probability of infection with VHSV in the sampled fish.

What is the best combination of criteria for targeted surveillance?

- Who? Fish that can be infected with VHSV (see Tier of Fish Species to be sampled – Table 2). Comprise the entire sample with Tier 1 and 2 species if possible, using the following guidelines to choose individual specimens. When priority Tier species are not available to make up the total sample, move to the next Tier group.
- Which moribund (sick) fish? Sick fish are preferred over healthy fish. Preferentially select fish displaying signs suggestive of VHSV or displaying signs of septicemia in general. Clinical signs might include exophthalmia, hemorrhages at base of fins, gills, eyes and skin, distended abdomen or abnormal swimming patterns such lethargy, flashing and spiraling. Next, select any moribund fish with signs that suggest a compromised immune system (e.g., trauma, stunted growth, lesions, etc.). These fish are more susceptible to viral infections. If Tier 2 or Tier 3 moribund fish with signs suggestive of VHSV or septicemia are available, they should be selected over apparently healthy Tier 1 fish.

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- Which healthy fish? Make up the remainder of the sample with randomly chosen apparently healthy fish, preferentially choosing first-feeding fry, or sexually maturing fish.
- How many? The sample size is 35 moribund fish or 170 apparently healthy fish. If not enough sick fish are present, select appropriate moribund fish, followed by enough apparently healthy fish to total 70 (single farmed species/year-class) or 170 (mixed or wild species/year-class).
- When? In the spring when water temperatures are rising, or in the fall when water temperatures are dropping. Ideally, aiming for water temperatures above 2˚C and below 20˚C.

Comment [k1]: Hi Lori, The original text is not clear enough. Can we make it simple? Or do you want to keep the surveillance value concept in for economic reasons (in which case a table may clarify your idea).

Susceptible Species

The list of species known to be susceptible to infection or disease with viral hemorrhagic septicemia (VHS) is evolving. For the purpose of this surveillance plan, VHSV and VHS, the disease caused by the virus, are considered synonymous. The Tier 1 and 2 species is closely harmonized with the revised USDA list of susceptible species for regulation [insert web link].

Comment [k2]: Hi Lori, I took out the bit about the Federal Order list because this table is not meant to follow it exactly. In addition, if we want to leave in the concept of anadromous with IVa, we have to include IVc.

To best allocate limited surveillance resources, species are prioritized from those with the greatest known effects from VSHV to those that have not been found with VHSV but may be at risk. Sampling of VHSV susceptible species for surveillance purposes will be prioritized as follows. Any endangered or locally threatened wild populations of fish should be excluded from this surveillance plan, regardless of their listed Tier status.

- Tier 1 species, the highest priority, have documented mortalities caused by VHSV IVb in Canada or the U.S.
- Tier 2 species have been documented with VHSV IVb isolations but no mortalities were attributed to VHSV IVb. These species should be collected when Tier 1 species are not available.
- Tier 3 species have been documented with other strains of VHSV, but not yet with IVb. These species could be collected from watersheds targeted for surveillance if Tier 1 and 2 species are not available.
- Tier 4 species have not yet been documented with any strain of VHSV, but are of research interest due to their taxonomic relationship to other susceptible species, or their extreme mobility throughout Great Lakes watersheds. For surveillance purposes, Tier 4 species should only be sampled if fish from Tiers 1-3 are not present in targeted waters.

New species will be incorporated into appropriate Tier categories, or the Tier classification will be adjusted as new detections of VHSV are found and new information on the disease becomes available.

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Table 2. Tier-level classification for targeted sampling of North American species known or suspected to be susceptible to VHSV. Species are prioritized from those with the greatest known effects from VSHV (Tier 1) to those that have not been found with VHSV but may be at risk (Tier 4). Fish to be sampled are preferentially selected from Tier 1, then Tier 2, followed by Tier 3, and finally Tier 4 if no susceptible species are available. Any endangered or locally threatened wild populations of fish should be excluded from this surveillance plan, regardless of their listed Tier status. This table will be updated as more information becomes available.

Tier	Documented by virus isolation and/or RT-PCR
1 VHSV IVb (freshwater genotype) positive, with mortalities or clinical infection	Bluegill Freshwater drum Gizzard shad Lake whitefish Muskellunge Rock bass Round goby Smallmouth bass Walleye White bass Yellow perch
2 VHSV IVb (freshwater genotype) positive, but no mortalities or clinical infection attributed to VHSV IVb	Black crappie Bluntnose minnow Brown bullhead Brown trout Burbot Carp Channel catfish Chinook salmon Emerald shiner Largemouth bass Northern pike Pumpkinseed Rainbow trout Spottail shiners Shorthead redhorse Silver redhorse Trout-perch
3 IVb (freshwater genotype) negative, but have been positive for other VHSV strains	Brook trout Coho salmon Golden trout Lake trout Mummichog Pink salmon Rainbow trout Shiner perch Striped bass Three spine stickleback
4 Phylogenetically related to VHSV susceptible species, or highly mobile by natural migration or human activities. None of these species has been VHSV positive to date. This tier is included in surveillance sampling only if fish from tiers 1,2 and 3 are not present in targeted waters.	American eel Chubs Clupeids – Alewife Other Coregonids – Bloater Fathead minnow Lake herring Lake trout Longnose sucker Sea lamprey Sunfish *Lepomis spp.* White crappie White sucker

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Appendix 4: Field Protocol for Sample Collection

Version 1.0 of the Standard Operating Procedures: Field Sampling for Viral Haemorrhagic Septicaemia Virus (CFIA) was modified for this surveillance document. Version 1.0 was developed specifically for the spring 2007 sampling effort to meet the requirements of the US-Canada Bilateral Initiative for Viral Haemorrhagic Septicaemia (VHS) in the Great Lakes Basin, CFIA Operations, DFO National Aquatic Animal Health Laboratory System, Ontario Ministry of Natural Resources, Quebec Ministry of Natural Resources and Wildlife, and Quebec Ministry of Agriculture, Fisheries and Food. Please note that the appendices to this document are not included but are available from the Aquatic Animal Health Division, CFIA. The field sampling protocols will be reviewed and revised before each fall and spring sampling period.

STANDARD OPERATING PROCEDURES
FIELD SAMPLING FOR VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS

MAY 2007
VERSION 1.0
CANADIAN FOOD INSPECTION AGENCY

VERSION 1.0

Authority:

CFIA Operations and NAAHP Aquatic Animal Health Division are responsible for the information contained in these standard operating procedures. All staff conducting field sampling under the authority of the Health of Animals Act are responsible for ensuring that the SOP is carried out properly.

These SOPs do not supercede any routine human health and safety procedures. All requirements of the Workman's Compensation Board must be observed. MSDS information for all chemicals likely to be used in these procedures is found in a separate document.

All activities must consider welfare of the fish being handled and sampled. CFIA is a signatory to the Canadian Council on Animal Care and as such must abide by animal welfare requirements. The guidelines for the use of fish in research, teaching and testing are included at the end of this document in Appendix 5.

The document has been reviewed by DFO Aquatic Animal Health Science, DFO National Aquatic Animal Health Laboratory System (NAAHLS) staff, representatives from Ontario Ministry of Natural Resources, Ministère de l'Agriculture, Pêcheries et Alimentation Québec (MAPAQ), and Société de la faune et des parcs Québec (FAPAQ).

Biosecurity

1.1 Staff Procedures:

Rationale:

In order to minimize the risk of inadvertent spread of pathogens from one location to another, proper hygienic precautions will be implemented.

The goal of this procedure is to decrease the risk of pathogens being transferred between sampling sites by staff members.

Equipment Required:

Disinfectant (MSDS information provided in Appendix 4)
Spray bottle for disinfectant
Sealed container (bucket or Tupperware container) for disinfectant footbath
Long handled plastic foot brush
Hand sanitizer (alcohol based hand cleanser)
Clothing that can be disinfected or discarded (e.g. raingear or disposable coveralls)
Rubber boots
Latex or nitrile gloves
Sealed garbage container

Order of site visits:

Whenever possible, staff should not visit more than one site in a day. If this is unavoidable, staff members must take hygienic precautions and plan the site visits to minimize the risk of pathogen spread.

Staff Hygienic Procedures:

For hatcheries, use footbaths and hand washes upon entering and exiting a sampling location. Ensure that footbaths are contained so they are not discharged into fish bearing waters.

Wear gloves when handling fish. Dispose of gloves in sealed garbage container between sampling locations.
Disinfect raingear after completing sampling by spraying rain pants and jackets with disinfectant.

Carry equipment and supplies to disinfect hands and footware between sites.

1.2 Equipment:

Rationale:

Equipment has the potential to spread infectious agents. Equipment is to be kept clean at all times and disinfected after use to limit pathogen spread.

Equipment procedures:

All material taken into the field for sample collection should be new or decontaminated to ensure that no fish pathogens are transferred to a new location. Especially when travelling

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from a high risk area to an area of lower risk, ensure that vehicles, boots, coolers, outerwear (such as boots, rain gear), etc do not act as vectors.

Cooler disinfection:
Coolers, as well as other equipment used, must be cleaned, disinfected and dried between uses to prevent cross contamination between sites or fish.

Clean equipment with hot soapy water; ensure all organic matter and debris has been removed. Allow a minimum of 10 minutes contact time with the soapy water. Smaller pieces of equipment can be submerged; larger pieces of equipment can simply have contact with foamy lather for 10 minutes.

Rinse equipment with clean tap water.

Spray equipment down with disinfectant at manufacturer's recommended concentrations and contact times.

Rinse disinfectant off equipment after 10 minutes and allow to dry prior to re-use.

Disinfectant protocols:

Use disinfectants according to manufacturer's directions (manufacturer's information is contained in Appendix 1of Appendices to VHS Field Sampling Protocols).

Maintain disinfectant concentration either by checking concentration or regular renewal of the product.

Dispose of disinfectants according to manufacturer directions and in a manner that meets the requirements of waste management regulations. Dispose of spent disinfectants away from fish bearing waters, if possible into a municipal sewer.

1.3 Vehicles and Vessels:

Vehicles:

Use clean vehicles for transport to sampling locations.

Ensure floor mats are rubber to allow disinfection at the end of the day's sampling.

Designate clean and dirty areas within the vehicle and maintain separation of clean and dirty equipment within the vehicle.

Package any materials that cannot be easily disinfected, such as coveralls and cotton gloves, in sealed plastic bags to be discarded or laundered at the office.

At the end of the sampling day (upon return to the home base), clean the 'dirty' area and floor mats by vacuum or brush out any organic matter.

Disinfect hard surfaces.

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Wash the outside of the vehicle with hot soapy tap water and spray down tires and wheel wells with disinfectant; allow a minimum of 10 minutes contact time with the disinfectant prior to rinse with fresh tap water.

Vessels:

Boats used in fish handling procedures can become contaminated with organic matter containing VHS virus.

Use clean boats to travel to fish sampling sites and follow the order of site visit guidelines in section 1.1. Ideally boats should be dedicated for sampling within the same water body.

Otherwise, at the end of each sampling day, scrub down the boat with soapy tap water to remove all organic matter. Spray down all hard surfaces (particularly areas below the water line when boats are hauled out of the water and any surfaces that have had contact with fish within the boat) with disinfectant and allow a minimum contact time of 10 minutes prior to rinsing with fresh tap water.

2.0 Collection of Fish

2.1 Sampling Sites:

Sampling sites will be pre-determined by the field coordinator. Both the coordinator and the field sampling crew will have discussed what species, numbers which are likely to be present to make up the fish sample, and how many visits are required to achieve the sample size. For unforeseen circumstances, more information is provided below and in the Decision Tree (section 2.6) to help assist the field sampling crew.

Fish should not be sampled in water temperatures that exceed the permissable threshold (this should be determined jointly with the Competent Authority). VHS virus is generally less likely to be isolated at temperatures greater than 15ºC and fish handling at elevated temperatures is very stressful for the fish.

2.2 Fish Identification:

Fish that cannot be identified in the field should be discarded and not included in the sampling.

2.3 Field Collection Information:

Sample sizes and sampling strategy to be determined jointly with the Competent Authority.

If sick fish are present and they have signs that are consistent with VHSV (see Section 2.4 of this document for a list of clinical signs consistent with VHSV), this event should be reported as soon as possible to the field coordinator or the appropriate provincial authorities. The event will then be considered a disease outbreak investigation and

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sampling will proceed according to the disease outbreak investigation protocols attached to this document.

Any fish appearing to be sick must be collected and placed in a separate bag labelled as 'sick'. Dead fish should only be collected if they are fresh (i.e. still showing redness in the gills) and must be bagged individually and labelled 'dead'.

Ideally, fish should be collected on a Monday or Tuesday to ensure samples will be processed and arrive at DFO's diagnostic labs before the weekend.

If more than one sampling visit is required to collect the required number of fish, fish collected on the first visit should ideally be sent to the staging laboratory. In the event that this cannot be done, fish can be held alive in a holding pen until the sample size is achieved. Different species of fish do not need to be held in separate pens.

2.4 Clinical signs consistent with VHSV:

Fish exhibiting any of the following signs may be infected with VHSV. Usually more than one clinical sign is present. However, in some species of fish, only hemorrhages of the skin may be present. The presence of these clinical signs DO NOT mean that fish are infected with VHSV. Laboratory testing IS REQUIRED.

large scale mortality (observation of > 5 dead fish at the sampling site)
large scale morbidity (observation of > 5 sick fish at the sampling site)
exophthalmia (pop-eye) of one or both eyes
hemorrhages at base of fins
hemorrhages in the gills
hemorrhages in and/or around the eyes
hemorrhages on the skin
abnormal swimming patterns such lethargy, flashing and spiraling
distended abdomen

2.5 Sample submission information required:

Date of Collection:
Location:
Species:
Total Number of Fish:
Reason for Submission: (e.g. surveillance or validation)
On Site Observations:

Sample submission information required on/in each bag of fish:

Species
Type of fish (sick, dead, or normal)
Date and time of collection

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All labelling must be done to ensure readability at the receiving end. This can be done by including the information written with a permanent marker on waterproof paper.

2.6 Decision Tree for Sampling Targeted Fish Species in a Watershed

Contact the Competent Authority for specific information regarding examples of a decision tree and what elements should be incorporated into the tree.

2.7 Finfish Euthanasia for fish being sampled:

Background:

Euthanasia is defined as a rapid and irreversible loss of consciousness that lasts until death, with no pain or distress accompanying the procedure. According to the CCAC[17] guidelines and definitions:

112: Where feasible, the euthanasia of fishes should consist of a two-step process, with initial anaesthesia to the point of loss of equilibrium, followed by a physical or chemical method to cause brain death.

113: If a physical technique of euthanasia is used when killing fishes, it should entail the physical destruction of brain tissue by pithing or crushing the brain.

List of equipment:

Disinfectant for gear (boots, raingear, coolers)
Anaesthetic
Plastic container to anaesthetize fish
Outer-wear including gloves for handling fish

Finfish Euthanasia guidelines:

Chemical Euthanasia:

Finfish anaesthetics require a veterinary prescription.

A two step method of euthanasia is the preferred method employing an anaesthetic overdose followed by a physical method of euthanasia (blunt force trauma to the head, pithing or decapitation).

Follow fish handling guidelines and anaesthetic protocols.

Details of the operating procedure:

Make a euthanasia bath.

Buffer the euthanasia bath if required.

[17] Canadian Council on Animal Care (2005) Guidelines on the care and us of fish in research, teaching and testing. Ottawa, Canada: Canadian Council on Animal Care.

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Gently transfer the fish from a sedative bath or holding tank into the euthanasia bath.

Monitor the fish until opercular movements stop.

Allow the fish to remain in the anaesthetic bath for 5 minutes after cessation of opercular movements.

Physical Euthanasia:
Apply blunt force trauma to the head.

Records: Log drug use.

3.0 Packing and shipment of euthanized fish to the stage laboratory

3.1 Packaging Freshly Euthanized Fish:

List of Equipment:

Waterproof, new plastic bags of various sizes
Labelling pens to write on bags- must be waterproof and permanent
Waterproof paper and pencil
Duct tape to seal coolers
Coolers
Gel paks or ice

All fish sampled must be bagged individually with sample collection information included on or in the bag. Bags of fish can then be put inside a second bag. All labelling must be done to ensure readability at the receiving end. This can be done by writing on the bag with permanent marker or by including the information written in pencil or permanent marker on waterproof paper.

Double bag fish in heavy duty plastic bags and seal tightly with tie wraps. Do not include water in the sample bags. The outer bag should have a tamper-proof closure. All bags should be placed in leak-proof containers that are sealed to prevent tampering during transportation to the laboratory.

Bag and tag fish according to their health status, healthy or appears sick, and attach identification to the bags. Bags are tied off in a knot.

Place gel paks or crushed ice in hard sided coolers to help keep fish cool (+4º±2ºC), but not frozen during shipment; cover the gel paks with newspaper or other shipping materials.

Place bags containing fish inside hard sided cooler and cover with newspaper or other shipping material.

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Inside cooler provide the following sample information, protected in a sealed waterproof plastic bag, for the staging lab:

Sample location
Date sampled
Fish species
Contact information for field crew (name and telephone)

Field crews should keep a copy of the information to pass on to the lab and for own records.

Apply tamper proof tape cooler to securely shut to prevent contamination and follow chain of custody procedures.

3.2 Shipping Fish to Staging Laboratory:

Transport methods suitable for shipping samples to the staging lab:

- by courier
- picked up and delivered by hand

Ensure the names and telephone numbers of couriers and the staging laboratory are readily available to field staff. Call the staging laboratory prior to sample shipment so they are prepared to handle the samples.

Staging Laboratory Contact Information:

To be determined

Records: Field log sheets, courier records, chain of custody paperwork

4.0 Finfish gross examination and necropsy techniques at the staging laboratory

Rationale:

Consistency in conducting necropsy procedures will ensure that all important information and samples are collected as required.

4.1 Equipment Required:

Household or commercial bleach (sodium hypochlorite)
Scalpel handles (# 3 and # 4)
Scalpel blades (#10, 21, 22 or 23)
Forceps
Scissors
Latex or nitrile gloves

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3 x 7¼ inch sample bags
Self-sealing plastic bags
Electrical Tape or Parafilm
Gel packs or ice
Isopropyl Alcohol (90 – 95%)
Data Sheets
Pen, pencil, and permanent marker
Paper towels
Low lint tissues
Glass or other suitable container for immersing tools
Source of flame (lighter, tealight or Bunsen burner)
Wire rack or equivalent
Disinfectant solution in spray bottle
Cooler with gel packs or ice

4.2 Preparation of Work Area:

Sterilizing work surfaces:

In between sample groups or before beginning, the work surface should be completely sterilized with a 0.25 % bleach solution that should be left on the surface for at least 10 minutes, before being wiped dry with a fresh piece of paper towel. A small stack of paper towel (around 4 pieces) is then placed on the clean work surface to act as a work surface between individual samples.

In between sampling individuals in a sample group, the carcass along with remaining organic material is discarded into an appropriate container for proper disposal. The work surface is subsequently sprayed with 0.25% bleach solution and then wiped clean with a paper towel. A fresh stack of paper towels is placed on the work surface for use with the next sample.

After a sampling period is finished the work surface should be wiped clean and sterilized as above before being stored in a clean, dry place.

Note: If dissection of tissues is conducted away from the sampling site (i.e. at a staging laboratory), all waste generated from a sampling period must be bagged, sterilized and disposed of properly (e.g. autoclaved, Sharps container, etc); this includes scalpel blades, paper towels, organic waste (carcasses), etc.

Sterilizing dissecting tools:

In between sample groups, the dissecting tools should be wiped clean of any organic materials with a clean piece of low-lint tissue, rinsed with water and soaked in a 0.25 % bleach solution for at least 10 minutes.

In between sampling individuals in a sample group, the dissecting tools (i.e. scalpel and scissor blades, etc.) should be wiped with a clean piece of low-lint tissue to remove any remaining organic material. Then the working ends of the tools should be immersed in a 5% bleach (household bleach) for 5 minutes, followed by a rinse in water, and finally

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immersed in 75% isopropyl alcohol followed by flaming to burn off the remaining alcohol. The tools can then be placed to cool on a wire rack (or equivalent).

Note: The solutions used above should be changed regularly as organic material can build up. Water should be changed frequently at least once every 10 individual samples.

4.3 Gross External Examination:

Visually examine the fish on both sides prior to laying it on the paper towel.

Note the general condition of the fish (e.g. presence of lesions, ulcerations, emaciated (excessively lean, bones clearly visible and coelomic cavity concave), deformed, or good body condition, etc).

Take digital photographs of fish with odd abnormalities with a label in the picture with sample information.

Lift the operculum to examine the gills for color, consistency and presence of any lesions. Gills should be bright red, note any gill pallor.

Lay the fish on the paper towel in right lateral recumbency.

Record observations on data entry form.

4.4 Sterile Technique:

When samples are collected for virology testing it is crucial to use sterile technique in sample collection. Contamination of instruments can result in false positive tests.

Open the fish with a sterile scalpel or scissors. After the incision is made, put the scalpel directly into an alcohol solution, and flame it prior to re use. Technicians may choose to use a new scalpel blade on each fish being sampled.

After each use the instruments are put into alcohol and then flamed before being put back into the fish being sampled.

Instruments are cleaned and disinfected prior to use. If possible they are autoclaved after a days use.

4.5 Gross Internal Examination and Sample Collection:

Using a sterile scalpel, open the fish with a simple incision on the ventral surface from the fill isthmus to the anal vent. This will allow quick access to the tissues necessary for virology.

Remove scalpel and forceps from the alcohol and flame prior to use on fish. Allow instruments to cool before touching them to the fish.

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Wipe tissue off instruments with paper towel and resubmerge in disinfectant. Flame instruments and allow to cool prior to opening body cavity.

Wipe organic matter from the instruments onto a clean paper towel and return them to the alcohol bath.

Flame instruments prior to reuse to ensure there is no contamination of the internal organs with bacteria from the outside of the fish.

Let instruments cool before touching them to tissues.

Use 3 x 7¼ inch sample bags for sampled tissues. Label sample bags with individual fish identifier prior to starting tissue collection.

The sample bags should be kept on ice while tissues are being collected to keep samples cool.

For diagnostic testing of surveillance samples, fish of the same species will be combined in pools of 5 fish or less.

4.7 Tissue samples for cell culture

Sterile technique must be used when collecting tissue samples.

Samples for viral isolation:

Amount and type of tissue to be collected for cell culture viral assays is determined by the age/size of the fish. See the table below.

Age/Size	Tissue	Quantity
Alevin	Whole fish with yolk sac removed	as much as possible
4-6cm	Entire viscera including kidney and heart	as much as possible
>6cm	Kidney and spleen (3:1 ratio), heart	0.5 to 2 grams
Brood Fish	Kidney and spleen (3:1 ratio), heart	0.5 to 2 grams

Cell culture for surveillance samples:

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For testing of surveillance samples, fish of the same species will be combined in pools of 5 fish or less.

Use 3 x 7¼ inch sample bags for sampled tissues. Label bags with pool identifier prior to starting tissue collection.

Once samples have been collected seal the sample bag and place in a cooler or refrigerator until the samples are packed and shipped to the recipient DFO laboratory.

These samples must be kept at 4–7°C and ideally they should be sent to the DFO labs within 24 hours of collection.

5.0 Packing and Shipment Procedures of Fish Samples to the Diagnostic Laboratories

5.1 Chain of Custody Procedures:

Rationale:

Without chain of custody procedures in place all samples collected are worthless for legal procedures. The chain of custody procedure incorporates a number of controls to assure the integrity of a sample.

These procedures, along with the required written documentation, provide the necessary backing to defend the integrity of the sample. The chain of custody procedure starts with sample collection and follows through to the destruction of the sample. The purpose of the procedure is to ensure that the sample has been in possession of, or secured by, a responsible person at all times. It should remove any doubt about sample identification or that the sample has been tampered with.

5.2 Details of the Operating Procedure

Sample Number:

Assign a number to all samples and date them, the number follows the sample through collection, transport and processing

Sample Tag or Label:

Attach to every sample container a tag or label with the following information written in waterproof ink: sample number, fish species, number of fish if a pooled sample, location where sample was taken, date and time of collection, what preservation is used, and your initials. Sample containers are often sealed with a tamperproof seal at this point.

Field Notebook:

Record all basic information such as the time and date of sampling, types of samples collected, personnel present and any other information that relates to the sampling event.

Chain of Possession Documentation:

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The form is filled out at sample collection and follows the sample through every person involved in the chain of possession until it reaches the laboratory. It includes information such as location of sampling, preservative used in containers, type and size of container for each sample, dates and times of collection, type of sample and the name of person collecting the sample.

Every time the sample changes possession, the person relinquishing the sample and the person receiving it must sign and date/time the Chain-of-Custody form. For example, the sampler may relinquish the sample to a courier. At the transfer, both parties sign and date/time the form. Then the courier delivers the sample to the laboratory where now the courier and lab representative sign and date/time the form.

5.3 Shipping Samples to Diagnostic Laboratories:

Samples may be rejected by the virology lab, if

a) at arrival in the virology lab, samples are older than 60 hours from collection of fish,

b) do not meet the temperature requirements (not frozen and not exceeding 7 °C during transit,

c) if the lab has not been notified in advance to allow for cell culture preparation. Because live cell cultures need to be prepared 24 hours ahead of sample processing in the virology lab, the receiving lab must be notified as early as possible and not later than 24 hours in advance of arrival . If samples are to be received on a weekend, notification of possible sample shipping must be received by noon on the Friday preceding the shipment.

Information specified below must accompany the samples.

Materials needed:

Sample bags
Coolers or other sturdy waterproof container for shipping
Gel-type ice packs or wet ice
Waterproof paper
Waterproof marker
Tupperware or similar containers with water proof lids
Temperature Probe
Clear plastic packing tape (Tamper-proofing tape)
Labels for outside of shipping container: KEEP COOL. DO NOT FREEZE

Instructions: Notify receiving lab at least 24 in advance estimated time of arrival. For weekend arrival times, labs must be notified by noon on Friday

Contact information is given below.

The following information must be included in the notification:

- Waybill number or Courier tracking number
- Estimated Time of Arrival,

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- number of samples to be processed. When in doubt, over estimating the number of samples is preferred to underestimating.
- Date and time of collection of fish in the field (= approximate time of death of fish)

Packing of samples into Tupperware container:

Disinfect thermometer module.

Notify receiving laboratory that samples are coming and include information on the number of samples being shipped and the weigh-bill number and estimated time of arrival

Pack Tupperware container with sample bags containing the tissue sample and information required by laboratory (**Ensure that the shipment includes an unused whirlpak/sample bag with each shipment (*necessary for receiving testing labs to tare balance prior to processing of tissue samples*))**

For each pool of fish, the following information must accompany the samples to the lab:

Case #
Sample#
Species
Number of fish in the pool and where samples are used for validation testing, individual fish numbers are required
Date and time of collection of fish

Information can be written directly on each sample bag with a waterproof marker or be written on waterproof paper

Place thermometer module into middle of bagged samples

Buffer material in the container by adding clean paper-towel if necessary.

Packing of cooler and shipping information:

Layer cooler with solidly frozen ice-packs. Place Tupperware container into the middle of the cooler and surround container(s) with clean paper-towel to act as buffer. Add another layer of icepacks.

Note that the tissue culture samples must not be frozen and the tissue culture samples should not exceed 7 ^{o}C at all times during transit.

Close cooler and fasten lid securely, adding taping or other means to ensure that container will show any opening in transit (tamper proofing). Clean plastic tape with signature across the end piece works well.

Ship by courier or by priority air cargo.

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Place label: KEEP COOL. DO NOT FREEZE on outside of shipping container together with address and contact person of receiving lab.

5.4 Log in at Laboratory:

Once received at the diagnostic laboratory the samples are logged in and their integrity checked for:

- Samples arrived within 60 hours
- Correct preservation
- Tamperproof seals intact
- Correct signatures present
- Min/Max temperature readings
- Samples do not appear to be decomposing (smell test)

Samples are accepted by the diagnostic laboratory, if the above conditions are met, as per laboratory sample acceptance SOP and the lab assigns its own ID number. If any of the conditions are not met, the diagnostic lab will refuse to process the samples because sample integrity is questionable.

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Appendix 5: Laboratory Protocols

Uncontrolled version to be submitted by DFO when completed.

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Appendix 6: Expert Panel

COMBINING SURVEILLANCE AND EXPERT EVIDENCE OF VIRAL HEMORRHAGIC SEPTICEMIA (VHS) FREEDOM: A DECISION-THEORETIC APPROACH

Abstract excerpted from an article prepared for submission to Preventive Veterinary Medicine by

L Gustafson, K Klotins, S Tomlinson, G Karreman, A Cameron and A Scott.

The current draft of the full manuscript is available upon request.

ABSTRACT

Combining multiple evidence streams to establish disease freedom or distribution is important for emerging diseases such as VHSV IVb in freshwater systems of US and Canada. Because waterways present a relatively unconstrained pathway for the natural distribution of VHSV, surveillance zones are defined by watershed (rather than geopolitical) boundaries. However, evaluating disease status at the level of the watershed has its challenges. We consider those challenges, and introduce a decision-theoretic approach to estimating watershed risk of VHSV infection that circumvents many of these issues. Information derived from historical evidence and expert opinion is used to supplement data derived from State/provincial and Federal surveillance resources. Field surveillance results and uncertainty are described with a beta distribution. Expert opinions on risk are solicited and summarized in the form of subjective likelihood ratios. Elicited likelihood ratios are derived by asking experts to individually estimate the predicted occurrence of a risk factor among VHSV-affected vs. VHSV-unaffected watersheds. The collective weight of risk factors describing a given watershed is represented by the product of applicable likelihood ratios. Finally, a simple Bayesian model multiplies the results from expert and field data streams to predict the probability of watershed VHSV-infection. This decision analytic approach yields a spatial risk-metric amenable to variations in field sampling intensity, yet comparable over jurisdictions and time.

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Appendix 7: Event Investigations (Canada only)

Disease Investigation Protocols For US-Canada Bilateral Initiative for Viral Haemorrhagic Septicaemia (VHS) in the Great Lakes Basin Spring 2007 Version 1.0

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Purpose

The purpose of this document is to assist field staff with the collection of samples associated with fish kills in wild fish populations during the spring and fall 2007 phases of the US-Canada joint initiative for Viral Haemorrhagic Septicaemia (VHS). These protocols are written for the field response after one of the regulatory authorities has been contacted about a fish kill (e.g. OMNR, DFO, CFIA, MAPAQ). Note that in general, these protocols for disease investigation will apply to any wild fish kill, as all fish kills are to be approached in the same manner.

Background

Fish kills are any unusual and noticeable or measurable increase in mortality and/or morbidity in one or more fish populations in a defined time period and geographic area. Mortalities in multiple species in the Great Lakes in 2005 and 2006 have been associated with the presence of the VHS virus (VHSv). Detection of the virus represents a disease control challenge due to the large number of susceptible species and the uncontrolled movement of water, fish, equipment and vessels throughout the Great Lakes. However, although many of the die-offs have been associated with detection of VHSv, it is not known whether VHSv is the only factor causing these die offs[18]. Doing more rigorous investigations of fish kills during the spring of 2007 will enable us to understand more about the disease and how it can be managed. This is important to many stakeholders who depend on the fisheries, for example bait fishermen, aquaculture producers, recreational and commercial fisherman and provincial fish stocking initiatives.

Investigatory Approach

At the site of the fish kill it is important to be open-minded, make clear and unbiased observations, listen carefully to any verbal information provided by observers, and collect as much information as possible. Often repeat visits, repeat sample collection, gathering further history about the event, getting on and in the water and persistence are necessary for successful disease investigations. What's most important is to get a good history with as much information about the event as possible. This involves being open-minded and asking open-ended questions -

[18]Fish kills are alternately called "die-offs", "epizootics" etc. In the Great Lakes they have been associated with other causes. For example, see http://www.miseagrant.umich.edu/downloads/habitat/botulism-FAQ-030107.pdf regarding botulism. Also, see "Causes of fish kills in wild fish" at the end of this document.

i.e. questions should not be leading or loaded - even if the cause of the fish kill appears obvious. There may be underlying factors involved that are not immediately apparent. (See "Causes of fish kills in wild fish" at the end of this document).

Key information

The following information should be gathered and recorded on the lab submission form.

- WHO
  - *Define the groups of animals affected*
    - Species affected (or possibly affected)
    - Size, age and life stage of the affected fish
    - Other vertebrates or invertebrates possibly affected (aquatic and terrestrial)
    - Other species of fish present in the area that appear to be unaffected

- WHAT
  - *Clearly define the problem and determine the magnitude/extent of the outbreak. Look not only at the dead animals but also what is happening to other species and the environment surrounding them.*
    - Clinical signs of disease
      - External observations (e.g. red fins, protruding eyes (exophthalmia), other visible lesions, excess mucus, etc.)
    - Estimate the number of dead and moribund fish (daily and cumulative mortality)
    - Observations of the animals environment such as water discoloration or peculiar clarity, turbidity, particulate matter etc., and general conditions such as time of day or night, water temperature precipitation, etc.

- WHEN
  - *Orient the fish kill in time. Understanding the time sequence of an outbreak can provide many important clues about the origin of the kill.*
    - Current estimated status of the fish kill, if possible. This would be based on information such as the condition of dead fish (i.e. freshly dead vs. severe decomposition).
    - Duration of the event, when was it first noticed, is it ongoing
- WHERE
  - *The geographical location and pattern of disease. Use a map or chart of the area and waterways if available.*
    - Fish kill location/watershed ID (preferred data: (latitude and longitude taken from a GPS (in decimal degrees))
    - Location fish kill initially noted
    - Any geographic pattern regarding observations of mortalities
    - Proximity to surrounding infrastructure, industry, roads, homes, sewage outfalls, storm sewers, enhancement hatcheries etc…

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        - Any other potential pattern in the area in which the event occurred – i.e. localized vs. broad geographic distribution of mortalities

- WATER QUALITY
    - *Recording water quality is essential to the investigation*
        - Water temperature (at surface and depth)
        - Dissolved oxygen level (at surface and depth)
        - pH
        - Location and depth of any water samples collected for phytoplankton or contaminant analysis
        - Water quality parameters– turbidity, clarity, colour, odour

- GENERAL HISTORY
    - *Ask any additional questions to help to clarify the situation.*
        - Recent environmental variations or other extremes
        - Local seasonal effects (if any)
        - Recent industrial or fishing practices in the area
        - History of fish kills in this area
        - Treatments used in the vicinity (i.e. agricultural fertilizers or herbicides)
        - Recent introductions of fish to this waterway
        - If known, populations in the waterway tested previously for VHSv, along with the dates and results of all tests.
        - Temporal pattern of mortality– is it day or night after rainfall or prolonged warm spell/cold spell etc.

- CONTACTS
    - *If they are willing to provide it, get contact information from all people with information regarding the fish kill. (Remember, their input is voluntary)*

The HOW and WHY are determined by laboratory investigation, in conjunction with the observations from the field and input from experts.

Recording information on the lab submission form

All samples must be properly labelled with the case number, location, species, date and name of collector. A proper lab submission form must be developed with the appropriate laboratory and approved by the appropriate regulatory authority prior to any fish kill investigation. (The lab submission form must be fully completed with all information available, and should be completed in pencil or waterproof ink).

Field procedures

Arrive at the site of the reported fish kill as soon as possible. It is imperative to make site observations, to get as much information as possible and to collect the best quality samples for laboratory testing as soon as possible. Lab testing will include bacteriology, virology,

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histopathology, toxicology and other tests as deemed appropriate to the situation by the regulatory authorities.

For this initial phase of the bilateral VHS initiative no dissection of fish will be done in the field. All fish for laboratory submission will be submitted dead on ice in coolers and shipped or transported immediately to the appropriate fish health laboratory. If VHS is suspected, the laboratory will be responsible for sending samples to the federal VHS Reference Laboratory (Pacific Biological Station, Nanaimo, British Columbia) to obtain a confirmatory diagnosis.

Care must be taken to get diagnostic quality fish to the lab as soon as possible. Poor quality samples should not be sent as diagnosis of any causative agent is very difficult with poor quality samples.[19] Place generous amounts of ice (or equivalent dry freezer packs) in the cooler to be sure that fish are well iced and kept cold for the duration of transport to the laboratory. Unfortunately, the VHS virus does not survive transport well, and fish can rapidly decompose during transport, particularly at high temperatures. Also, unforeseen circumstances that arise during transport can result in prolonged time for the specimen to reach the laboratory.

Before you go out in the field, ensure you are familiar with all the required biosecurity measures and the field procedures for collection, packaging and sampling as described in Appendix 4: Standard Operating Procedures Field Sampling for Viral Haemorrhagic Septicaemia virus (Sections 2 and 3). You must be familiar with these protocols and have the proper equipment and supplies with you *before* leaving for the site. If possible take along a digital or video camera that records date and time to help document the site observations.

Selecting fish for sampling

Whole fish that are collected are to be bagged individually and sent on ice to the laboratory.

Number of fish

- A minimum of 10 diagnostic quality specimens is required when a single fish species and age class all with similar grossly observable signs appears to be affected.
- In cases where there are multiple fish species or age classes affected, or a variety of clinical signs present, a cross section of samples should be collected from the various populations up to a maximum of 35 fish in total. This should be a combination of species, age classes and sizes showing various levels of disease signs that represent as closely as possible what is observed at the site of the fish kill.

Moribunds vs. mortalities

[19] If *only* poor quality samples are available then contact the Coordinator immediately for guidance on sample collection.

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- Moribund fish are preferred if available, as these are generally the best samples for determination of the causative agent[20]. They can sometimes be difficult to obtain but should be collected immediately.
- If there are insufficient numbers of moribunds available, fresh dead specimen are also acceptable. Fresh dead fish can be identified by: lack of strong, pungent smell; flesh still feels firm; and fish remain intact when handled.
- However, if no suitable specimens are available water samples should still be collected and the information on the fish kill gathered.
- Contact the appropriate regulatory authority regarding the necessity of follow-up visits to the site for specimen collection. Follow-up visits may need to occur on a regular basis until sufficient samples have been collected or the investigation has been closed.

Euthanasia

- For euthanizing live or moribund fish for health testing follow the procedures outlined in Appendix 4: Standard Operating Procedures Field Sampling for Viral Haemorrhagic Septicaemia virus (Section 2.5)

Water quality

The water at the site should be tested for dissolved oxygen, pH and temperature as soon as possible upon arrival at the site. The measurements should be taken in the location of the largest number of observed mortalities. Ensure the location and depth of where the water measurements are taken is included in the lab submission form.

The water samples should be collected from areas where the water appears abnormal in color, clarity or smell. If unusual, bothersome smells or sensations are encountered such as but not limited to watering eyes, tingling skin or respiratory distress you should leave the area immediately and contact the proper authorities.

Three samples of water for analysis of pesticides, solids and phytoplankton must be collected. Make sure the location and sample depth as well as the time and date are entered in the lab submission form. Ensure also that the sampling containers are suitable for water quality analysis, and do not contaminate the water sample. Please refer to your respective agency's sampling protocols and see Langdon (1988)[21] for more information.

Do not smoke during the water sample collection as this can interfere with the analysis.

[20] Moribunds are preferred in cases where there is high index of suspicion regarding the cause (in this case VHS) however moribunds may not be the preferred samples in other situations.

[21] Langdon, J. (1988) Investigation of Fish Kills. I: History and Causes; II: Investigatory Approach. In "Fish Diseases: Refresher Course for Veterinarians." Proceedings 106: 23-27. May 1988. Post Graduate Committee in Veterinary Science, University of Sydney, Sydney, Australia.

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Findings

The purpose of investigating fish kills in spring and fall of 2007 will be to determine if there is a definitive pathological agent causing the fish kill (e.g. VHSv) or whether other factors are also involved. This is important for managing and predicting future losses due to the virus.

However, it should be noted that it is not uncommon, in this type of investigation, for the cause of the fish kill not to be fully understood. Often numerous visits and sample collection over a period of time are required before the causative agent can be determined. Follow up steps will be determined by the regulatory authorities after initial samples have been analyzed.

Professional attitude

All aspects of any disease investigation must be carried out in a professional manner. The public is concerned about VHS and expects a professional approach with regard to field investigations and information on disease spread and control.

Good communication and coordination must occur between the field personnel and the laboratories. The regulatory authorities will manage overall communications with the public.

Causes of Fish Kills in Wild Fish

The reason for the fish kill may not be obvious and it might not be due to just one cause. A complete and comprehensive disease investigation is required in order for accurate determination of the causative agent(s) of the fish kill. Often a multitude of factors are involved in fish kills. Fish kills can be a natural event such as Chinook salmon dying after spawning. Fish kills can also be caused by one or more combinations of infectious agents, natural and pollutant toxins, as well as environmental and climatic variations and extremes.[22] (Also see Langdon (1988)[4]). Fish kills in the Great Lakes may not necessarily be due to VHSv. For example in the past there have been fish kills in association with botulism (see link provided on page 1) and thiamine deficiency. Therefore, a full outbreak investigation is required in order to identify the ultimate cause of the mortality.

- **Infectious Agents**

  Many groups of pathogens have been implicated in fish kills. Mortalities due to infectious disease can be sudden and acute or chronic and spread out over time. The mortality event can display seasonal or diurnal patterns with single or multiple species affected. Pathogens when involved in fish kills are often the actual cause of death, however, they are seldom the sole cause.

[22] Often by the time the fish kills are reported the environmental incident has passed, which may be why the causes of so many fish kills are never resolved.

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- **Non-Infectious Biological Factors**

Algae are a common cause of large fish kills in the wild and a number of different algae species may be responsible. Some species of algae cause fish kills by depleting the water of oxygen, others cause mechanical damage to the gills of the fish, while other species of algae can produce toxins.

Non-infectious biological factors such as overcrowding and certain life cycle events may also lead to large fish kills. For example, post-spawning death of Chinook salmon is one example of a life cycle event which results in mortality.
Although fish kills associated with over-crowding are more common with aquaculture production, over-crowding can also play a role in wild populations during environmental shifts such as drought and low river flows.

- **Environmental Events**

Seasonal and climatic events can lead to fish kills. Extremes of rainfall or temperature can lead to mortality in a variety of fish species. For example, heavy rains can lead to run-off of silt into the water column which may lead to gill obstructions and death. Low temperatures may lead to host stress or may be directly lethal. High water temperatures may lead to host stress, oxygen depletion or be directly lethal. Different species have differing abilities to handle temperature fluctuations and extremes. Prolonged exposure to temperatures outside of the species' optimal temperature range is generally lethal.

Other weather related events such as strong winds due to hurricanes or tornadoes (water spouts) can cause physical trauma which leads to mortality in affected fish.

Catastrophic events such as tsunamis and earthquakes can also lead to large fish kills.

- **Pollutants**

A wide range of toxic pollutants have been implicated in fish kills. The toxicity of chemicals to fish varies with species, age, previous exposure history, simultaneous infectious diseases or toxin, and other host stressors.

For further information see Langdon (1988)[4].

- **Fishing or other Industry Related Activities**

A number of activities in the area of the waterway can lead to fish kills such as operations which lead to large depositions of silt and sand or water diversions which can lead to over-crowding and oxygen depletion.

- **Translocations**

Fish released in the waterway for such activities as enhancement can have high mortality upon release due to a number of reasons such as unfavourable conditions at transport, significant temperature or salinity change upon release.

- **Other Causes**

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The use of explosives in the waterway and electrocution (Langdon (1988)[4]) are just two of a number of other potential causes of fish kills.

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