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Development and Evaluation of a Blocking Enzyme-Linked Immunosorbent Assay and Virus Neutralization Assay To Detect Antibodies to Viral Hemorrhagic Septicemia Virus

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Viral hemorrhagic septicemia virus (VHSV) is a target of surveillance by many state and federal agencies in the United States. Currently, the detection of VHSV relies on virus isolation, which is lethal to fish and indicates only the current infection status. A serological method is required to ascertain prior exposure. Here, we report two serologic tests for VHSV that are nonlethal, rapid, and species independent, a virus neutralization (VN) assay and a blocking enzyme-linked immunosorbent assay (ELISA). The results show that the VN assay had a specificity of 100% and sensitivity of 42.9%; the anti-nucleocapsid-blocking ELISA detected nonneutralizing VHSV antibodies at a specificity of 88.2% and a sensitivity of 96.4%. The VN assay and ELISA are valuable tools for assessing exposure to VHSV.

Viral hemorrhagic septicemia (VHS) is one of the most pathogenic viral diseases of fish worldwide and affects a wide range of host species (1–7). Of the four genotypes, the North American strains of VHS virus are designated types IVa and IVb. Type IVa was originally isolated from asymptomatic marine salmonids in the Pacific Northwest in 1988 (8); it is now known to be endemic throughout the northeast Pacific, where it is highly virulent to populations of Pacific herring (*Clupea pallasii*) and other marine fishes (9). A new freshwater strain, type IVb, was isolated from a muskellunge (*Esox masquinongy*) collected from Lake St. Clair, MI, in 2003 (10). This distinctive sublineage has been isolated from 31 species of fish in the Great Lakes (11) and has been associated with significant die-off events of freshwater drum (*Aplodinotus grunniens*), muskellunge (*Esox masquinongy*), gizzard shad (*Dorosoma cepedianum*), round gobies (*Apollonia melanostomus*), and yellow perch (*Perca flavescens*) in the Great Lakes between 2005 and 2008 (2, 10, 12–16). By 2009, the virus had spread to all of the Great Lakes and several inland lakes. The introduction and spread of this pathogen and the threat it poses to a broad range of hosts resulted in increased surveillance of the virus in Wisconsin and other states within the Great Lakes Basin.

Currently, the surveillance methods for VHS virus (VHSV) detection include virus isolation in cell culture, followed most commonly by confirmation by reverse transcription-PCR (RT-PCR). Fish are tested for VHSV according to the guidelines outlined in the American Fisheries Society Fish Health Section Blue Book (17) and the World Organisation for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals (18). Both approved methods detect the virus but do not detect antibodies indicative of previous virus exposure.

Clinical signs of disease are not consistent among VHSV-susceptible species, and VHSV IVb is not always isolated from clinically affected fish, especially salmonids (19, 20). Differences in susceptibility and mortality rates among different populations of yellow perch have been reported recently (21). The clinical signs and severity of infection also depend on water temperature at the

time of infection, stress level, host age, and other environmental factors (15, 22). These variables can affect the narrow window of opportunity to detect VHSV by virus isolation; therefore, diseased or recovered individuals may easily be missed during surveillance efforts.

Methods to detect neutralizing antibodies to VHSV have been developed for surveillance using a complement-dependent neutralization test (50% plaque neutralization test [PNT]) and have been highly sensitive and specific for trout (23–25). However, PNT requires overlay and plaque enumeration steps; further, this method is best suited for small sample sizes. A microneutralization format without the use of overlay might lead to a 50% reduction in the resources and labor required to perform the assay. Another advantage of a virus neutralization assay is that the indicator system is a susceptible cell line for the target virus, which makes the assay inherently species independent.

Competitive and blocking enzyme-linked immunosorbent assays (ELISAs) for the detection of antibodies against mammalian viruses have been in use for decades. Indirect ELISAs have been available for VHSV since 1988 (26). A highly sensitive (92%) indirect ELISA for detecting nonneutralizing antibodies for the surveillance of VHS in farmed rainbow trout (*Oncorhynchus mykiss*) has also been described (25), but it requires a species-specific secondary fish antibody. Thus, these tests are not practical for multispecies VHSV surveillance in the wild, because there are at least 31 species known to be susceptible to VHSV IVb.

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Here, we describe the development and evaluation of a modified virus neutralization (VN) assay and a blocking enzyme-linked immunosorbent assay (ELISA). Both tests were adapted from previously described methods in the World Organisation for Animal Health (OIE) manual (27) and use blood serum samples from uninfected fish and VHS survivors. Although the development of the anti-VHSV nucleocapsid monoclonal antibody used in our ELISA was published in 1988 (26), no competitive ELISA methods have been reported until now. These serological methods broaden the window of detection by demonstrating whether previous exposure to the virus had occurred, which might alleviate the time constraints of surveillance efforts using virus isolation.

MATERIALS AND METHODS

Cell lines. The epithelioma papulosum cyprini (EPC) cell line (American Type Culture Collection, Manassas, VA), originating from fathead minnow (*Pimephales promelas*) epithelial cells (28), was cultured at 25°C according to detailed protocols (18, 29), and the medium was supplemented with tryptose phosphate broth (Teknova; Hollister, CA, USA), 5% fetal bovine serum (FBS) (PAA Laboratories, Inc., Etobicoke, Ontario, Canada), 200 mM L-glutamine (Life Technologies), and buffered with 7.5% sodium bicarbonate solution (Life Technologies). The Chinook salmon embryo (CHSE-214) cell line (American Type Culture Collection, Manassas, VA) was cultured at 20°C, and the medium was supplemented with 10% FBS (PAA Laboratories, Inc.).

Virus isolate. The Great Lakes strain of VHSV (type IVb) was isolated on the EPC cell line. The isolate was obtained from pooled kidney and spleen tissue samples from a freshwater drum in Lake Winnebago during a VHS outbreak in 2007 and confirmed by real-time RT-PCR (30, 31).

Virus propagation and purification. Virus was adsorbed to the EPC cells at a multiplicity of infection (MOI) of 0.01 for 30 min at room temperature and then supplemented with cell culture medium. To propagate virus, flasks were incubated at 15°C for 5 to 7 days or until the first signs of cytopathic effect (CPE) were observed. A plaque-purified stock of VHSV-infected EPC cell supernatant was clarified by the removal of EPC cells, aliquoted for one-time use, and stored at -80°C. Postfreezing, an aliquot was thawed, and the titer of the batch was determined.

CHSE cells were used to propagate virus for ELISA antigen coating. The flasks for propagation were inoculated using the same methods as for the EPC cells but virus was adsorbed for 1 h at room temperature. After two freeze-thaw cycles, cell debris was removed by centrifugation at 4,000 × g for 15 min at 4°C in a Sorvall ST40R centrifuge (Thermo) and clarified. The supernatant was purified and concentrated according to the manufacturer's protocols using a Fast-Trap virus purification and concentration kit (Millipore, Billerica, MA). Eluted virus was aliquoted and stored at -80°C. A mock infection was performed in a similar manner to provide cell lysates for determining an optical density baseline in uninfected CHSE cells. Antigen was treated with 10% MEGA-10 detergent (Sigma-Aldrich) for an hour at room temperature prior to diluting in coating buffer for use in the ELISA.

Sera from fish of known infection status. Blood serum samples were obtained from 33 uninfected fish (Table 1), including brown trout (*Salmo trutta*) and yellow perch. A blood serum sample with antibodies to spring viremia of carp virus (SVCV) was obtained from a common carp (*Cyprinus carpio*) (Table 1). The serum samples were collected 4 to 5 months after an SVCV epizootic occurred in May 2002 in Cedar Lake, WI, and tested positive for neutralizing antibodies to SVCV at the Center for Environment, Fisheries, and Aquaculture Science (CEFAS) in Weymouth, United Kingdom, using a competitive ELISA (32). Serum samples were obtained from 28 experimentally infected or wild-caught fish that had survived exposure to VHSV (Table 2), including grass carp (*Ctenopharyngodon idella*), yellow perch, Pacific herring (*C. pallasii*), muskellunge, and freshwater drum. All serum samples were stored frozen at -80°C and

TABLE 1 Virus neutralization and blocking ELISA results for VHS-negative group

Serum source	VN titer result	ELISA data	
		% inhibition ^a	Result
<i>Salmo trutta</i> (brown trout) 1 ^b	Negative	8.13	Negative
<i>Salmo trutta</i> (brown trout) 2	Negative	31.54	False positive
<i>Salmo trutta</i> (brown trout) 3	Negative	18.88	Negative
<i>Salmo trutta</i> (brown trout) 4	Negative	13.38	Negative
<i>Salmo trutta</i> (brown trout) 5	Negative	19.84	Negative
<i>Salmo trutta</i> (brown trout) 6	Negative	4.65	Negative
<i>Salmo trutta</i> (brown trout) 7	Negative	14.58	Negative
<i>Salmo trutta</i> (brown trout) 8	Negative	20.2	Negative
<i>Salmo trutta</i> (brown trout) 9	Negative	13.50	Negative
<i>Salmo trutta</i> (brown trout) 10	Negative	8.73	Negative
<i>Salmo trutta</i> (brown trout) 11	Negative	13.50	Negative
<i>Salmo trutta</i> (brown trout) 12	Negative	24.73	Negative
<i>Salmo trutta</i> (brown trout) 13	Negative	5.50	Negative
<i>Salmo trutta</i> (brown trout) 14	Negative	8.13	Negative
<i>Salmo trutta</i> (brown trout) 15	Negative	14.34	Negative
<i>Salmo trutta</i> (brown trout) 16	Negative	15.18	Negative
<i>Salmo trutta</i> (brown trout) 17	Negative	9.92	Negative
<i>Salmo trutta</i> (brown trout) 18	Negative	9.90	Negative
<i>Salmo trutta</i> (brown trout) 19	Negative	41.10	False positive
<i>Salmo trutta</i> (brown trout) 20	Negative	11.35	Negative
<i>Salmo trutta</i> (brown trout) 21	Negative	13.03	Negative
<i>Salmo trutta</i> (brown trout) 22	Negative	14.81	Negative
<i>Salmo trutta</i> (brown trout) 23	Negative	16.49	Negative
<i>Salmo trutta</i> (brown trout) 24	Negative	5.14	Negative
<i>Salmo trutta</i> (brown trout) 25	Negative	20.13	Negative
<i>Salmo trutta</i> (brown trout) 26	Negative	14.34	Negative
<i>Salmo trutta</i> (brown trout) 27	Negative	34.29	False positive
<i>Salmo trutta</i> (brown trout) 28	Negative	24.73	Negative
<i>Salmo trutta</i> (brown trout) 29	Negative	2.39	Negative
<i>Salmo trutta</i> (brown trout) 30	Negative	0.24	Negative
<i>Perca flavescens</i> (yellow perch) 1 ^c	Negative	12.66	Negative
<i>Perca flavescens</i> (yellow perch) 2	Negative	27.48	False positive
<i>Perca flavescens</i> (yellow perch) 3	Negative	23.90	Negative
<i>Cyprinus carpio</i> (common carp) ^d	Negative	31.45 ^e	Negative

^a Results determined positive at ≥25% inhibition for test sera diluted 1:2 and ≥35% inhibition for undiluted test sera.

^b Brown trout 1 to 30 were captive broodstock from Westfield, Wisconsin State Fish Hatchery that were never exposed to VHSV.

^c Yellow perch 1 to 3 were lab-reared at the Great Lakes Water Institute in Milwaukee, WI, and never exposed to VHSV.

^d Wild-caught from Cedar Lake, WI, following spring viremia of carp virus (SVCV) epizootic in May 2002. The serum sample was positive for neutralizing antibodies to SVCV at the Weymouth Laboratory, Weymouth, United Kingdom, using standard methods (40).

^e Result from ELISA with undiluted serum, in which the positive threshold is ≥35% inhibition.

then heated to 45°C for 30 min to inactivate complement before use in assays.

All yellow perch used in the study were hatched and reared at the University of Wisconsin, Milwaukee (UWM) School of Freshwater Sciences (SFS) Aquaculture Research Facility, according to previously described methods (33). They were exposed to VHS virus strain IVb (MI03) by intraperitoneal (i.p.) injection at a titer of 1 × 10⁴ PFU/fish (J-Z fish) or 1 × 10² PFU/fish (H-Y fish). At 28 days post-VHSV injection, all yellow perch exhibited mild clinical signs of the disease, such as exophthalmia and hemorrhaging. The fish euthanized on day 64 appeared healthy, exhibiting no clinical signs of VHS, and all plaque assay results were negative.

Hyperimmunized Pacific herring (*C. pallasii*) were produced from laboratory-reared specific-pathogen-free (SPF) colonies (34). Briefly, SPF herring ≥5 years of age were immersed in waterborne VHSV (1.5 × 10³

TABLE 2 Real-time RT-PCR, virus neutralization, and ELISA results for VHS-positive group

Serum source	VHS PCR result	VN titer result	ELISA data	
			% inhibition ^d	Result
<i>Ctenopharyngodon idella</i> (grass carp) 1 ^b	Negative	Negative	75.12	Positive
<i>Ctenopharyngodon idella</i> (grass carp) 2 ^b	C _T 39.5	Negative	58.38	Positive
<i>Perca flavescens</i> (yellow perch) HI-14 ^c	C _T 36.5	1:16	72.97	Positive
<i>Perca flavescens</i> (yellow perch) J2-13 ^d	Negative	p1:16 ^j	78.26	Positive
<i>Perca flavescens</i> (yellow perch) J1-13/J3-11 ^d	C _T 37.8	p1:16 ^j	46.96	Positive
<i>Perca flavescens</i> (yellow perch) H4 A ^c	C _T 35.7	p1:16 ^j	36.92	Positive
<i>Perca flavescens</i> (yellow perch) H4 B ^c	C _T 35.7	p1:16 ^j	54.04	Positive
<i>Perca flavescens</i> (yellow perch) H4 C ^c	C _T 38.6	Negative	55.93	Positive
<i>Perca flavescens</i> (yellow perch) J4 A ^d	C _T 32.6	Negative	42.30	Positive
<i>Perca flavescens</i> (yellow perch) J4 B ^d	Negative	Negative	27.62	Positive
<i>Perca flavescens</i> (yellow perch) Z1-2 ^e	Negative	Negative	81.42	Positive
<i>Perca flavescens</i> (yellow perch) Z2-1 ^e	Negative	Negative	95.99	Positive
<i>Perca flavescens</i> (yellow perch) Z2-2 ^e	Negative	Negative	57.22	Positive
<i>Perca flavescens</i> (yellow perch) Y1-2 ^f	Negative	Negative	49.47	Positive
<i>Perca flavescens</i> (yellow perch) Y3-1 ^f	Negative	Negative	47.60	Positive
<i>Perca flavescens</i> (yellow perch) Y3-3 ^f	Negative	Negative	56.15	Positive
<i>Clupea pallasii</i> (Pacific herring) 140 ^g	Negative	p1:16 ^j	42.22	Positive
<i>Clupea pallasii</i> (Pacific herring) 141 ^g	Negative	Negative	56.22	Positive
<i>Clupea pallasii</i> (Pacific herring) 142 ^g	Negative	p1:16 ^j	56.91	Positive
<i>Clupea pallasii</i> (Pacific herring) 143 ^g	Negative	Negative	41.23	Positive
<i>Clupea pallasii</i> (Pacific herring) 144 ^g	Negative	Negative	30.95	Positive
<i>Clupea pallasii</i> (Pacific herring) 145 ^g	Negative	1:32	27.23	Positive
<i>Clupea pallasii</i> (Pacific herring) 146 ^g	Negative	p1:16 ^j	43.88	Positive
<i>Clupea pallasii</i> (Pacific herring) 147 ^g	Negative	Negative	26.15	Positive
<i>Clupea pallasii</i> (Pacific herring) 148 ^g	Negative	p1:16 ^j	34.67	Positive
<i>Clupea pallasii</i> (Pacific herring) 149 ^g	C _T 38.9	Negative	41.82	Positive
<i>Esox masquinongy</i> (muskellunge) ^h	Negative	1:80	7.94	False negative
<i>Aplodinotus grunniens</i> (freshwater drum) ⁱ	Negative	1:16	32.32	Positive

^a Results determined positive at $\geq 25\%$ inhibition for test serum diluted 1:2.

^b Injected intraperitoneally (i.p.) with 200 μ l of 10^6 PFU/fish VHSV IVb, serum collected 21 days post-i.p. injection.

^c Injected i.p. with 1×10^2 PFU/ml VHSV IVb, held at 12°C, serum collected 28 days post-i.p. injection.

^d Injected i.p. with 1×10^4 PFU/ml VHSV IVb, held at 12°C, serum collected 28 days post-i.p. injection.

^e Injected i.p. with 1×10^4 PFU/ml VHSV IVb, held at 12°C, serum collected 64 days post-i.p. injection.

^f Injected i.p. with 1×10^2 PFU/ml VHSV IVb, held at 12°C, serum collected 28 days post-i.p. injection.

^g Hyperimmunized Pacific herring were exposed to 1.5×10^3 PFU/ml VHSV IVa by waterborne immersion for 1 h (day 0). The survivors were reexposed by i.p. injection after 49 days (2.9×10^2 PFU/fish) and 77 days (2.8×10^1 PFU/fish). Serum samples were collected from the hyperimmunized survivors after 112 days.

^h Survived infection with VHSV IVb. The reference serum was received already diluted at 1:20 and used as the starting dilution for the VN assay. A new aliquot was obtained and used at 1:2 in the ELISA.

ⁱ Wild-caught on Lake Winnebago in Wisconsin on 9 May 2012. Kidney and spleen tissues tested positive for VHSV by real-time PCR according to previously described methods (31).

^j p1:16, partial neutralization at this dilution.

PFU/ml) for 1 h. The survivors were reexposed to VHSV by i.p. injection after 49 days (2.9×10^2 PFU/fish) and 77 days (2.8×10^1 PFU/fish). Serum samples were collected from the hyperimmunized survivors 112 days after the initial waterborne exposure.

Virus neutralization. The VN assay to detect VHSV-neutralizing antibodies was modified from the mammalian VN assay protocol based on previously described methods (27). The VHSV VN assay was performed as follows: first, epithelioma papulosum cyprini (EPC) cells were pre-seeded onto sterile microtiter plates, typically 2 days prior to inoculation to achieve 100% confluence. Next, 50 μ l of $100 \times$ the 50% tissue culture infective dose (TCID₅₀) of virus (35, 36) was mixed with 2-fold serial dilutions of serum starting at 1:16 in 96-well cell culture microtiter plates (BD Biosciences, San Jose, CA) and incubated at 15°C for 24 h. A back titration plate with 10-fold dilutions of the working dilution of virus ($100 \times$ TCID₅₀) was included to confirm the correct virus concentrations. Serum controls (serum without virus) were performed for each sample as well as an antibody positive and negative control on each plate. The cells were treated with 7% polyethylene glycol (PEG) for 10 min (37). Lastly, serum-virus mixtures were inoculated onto the PEG-treated cells, cov-

ered, and incubated at 15°C for 5 days. The virus neutralization titers were read as the last serum dilution showing protection of the cell monolayer.

Cross-reactivity of anti-VHSV nucleocapsid monoclonal antibody with SVCV. Mouse anti-VHSV nucleocapsid monoclonal IgG antibody (Aquatic Diagnostics, Stirling, Scotland) (26) was purchased for use in the blocking ELISA. The specificity of the anti-VHSV nucleocapsid monoclonal antibody to VHSV nucleocapsid was assessed by performing a Western blot, as previously described (38, 39). Spring viremia of carp virus (SVCV) is a rhabdovirus that is closely related to VHSV and that also causes disease during the spring season. The lysates were obtained from an isolate circulating during an SVCV epizootic in wild common carp (*C. carpio*) in northwestern Wisconsin (40). VHSV and SVCV lysates were separated on 4 to 20% gradient gels by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad) using a wet transfer Mini Trans-Blot cassette according to the manufacturer's protocols in the Mini-PROTEAN Precast Gels Instruction Manual and Application Guide (Bio-Rad), with the following modifications. The membranes were soaked in Tris-buffered saline containing 0.05% (vol/vol) Tween 20 (TBS-T) with 5% StartingBlock (PBS) blocking buffer (Thermo) overnight at 4°C. The

membranes were then incubated with anti-VHSV nucleocapsid monoclonal antibody diluted 1:100 in StartingBlock (PBS) blocking buffer at room temperature for 1 h with constant agitation. Three 5-min wash steps with TBS-T were performed after each antibody incubation step. The membranes were then incubated with peroxidase-rabbit anti-mouse IgG (H+L) (Invitrogen) at 1:1,000 in StartingBlock (PBS) blocking buffer for 1 h. A CN/DAB substrate kit (Thermo) was used for chromogenic detection of horseradish peroxidase-bound antibodies and stopped with deionized water.

Blocking enzyme-linked immunosorbent assay. A blocking ELISA was developed using modifications to a previous ELISA method (41). The anti-VHSV nucleocapsid monoclonal antibody utilized in our assay was previously shown to lack neutralizing activity (26). The antibody was purified and conjugated to horseradish peroxidase (HRP) using a commercial laboratory (American Qualex, San Clemente, CA). Alternating rows of purified MEGA-10 detergent-treated VHSV antigen and mock-infected MEGA-10 detergent-treated antigen diluted 1:100 in carbonate-bicarbonate buffer (pH 9.6) (Sigma) were adsorbed to 96-well Immulon 2 HB microtiter plates (Thermo) for 24 h at 21°C in an EchoTherm IN20 incubator (Torrey Pines Scientific) and then blocked with 200 μ l StartingBlock (PBS) blocking buffer for 2 h at 20°C. Antigen and blocking buffer were aspirated from the wells using an ELx405 microplate washer (BioTek). Fifty microliters of fish test serum (either straight or diluted 1:2) was added to the wells containing VHSV antigen and mock-infected antigen and incubated for 30 min at 37°C. Directly after incubation (without washing or removal of test sera), 50 μ l of the HRP-conjugated monoclonal antibody, diluted 1:5,000 in StartingBlock (PBS) blocking buffer, was added to the wells and incubated with the test sera for an additional 90 min at 37°C. The plates were then washed 3 times with phosphate-buffered saline (PBS) (pH 7.2) containing 0.05% Tween 20 (Sigma) to remove unbound antibodies. PBS was made by diluting 18.46 g of FTA hemagglutination buffer (BD, Chicago, IL) in 2 liters of deionized water. Sure-Blue 3,3',5,5'-tetramethylbenzidine (TMB) 1-component microwell peroxidase substrate (KPL, Gaithersburg, MD) was used as an enzyme substrate and chromogen for development of the assay. One hundred microliters of enzyme substrate was added to each well, and the assay was developed for 15 min at 37°C. The reaction was terminated by adding 100 μ l of 1% HCl TMB Stop Solution (KPL, Gaithersburg, MD) per well and the optical density (OD) at 450 nm was measured in an ELx808 absorbance microplate reader (BioTek). Multiple modified checkerboard experiments were performed to determine the optimal working dilution for the HRP-conjugated monoclonal antibody and the antigen concentrations for the coating plates.

The serum samples were tested both undiluted and at a 1:2 dilution in PBS wash solution. All OD readings for the samples and controls were adjusted by subtracting the background OD levels in the mock-infected wells. The percent inhibition (%I) was calculated using the formula $\%I = 100 - (100 \times \text{sample}_{OD} / \text{negative control}_{OD})$.

The presence of blue color after incubation with enzyme substrate indicated an absence of anti-nucleocapsid antibodies in a well. A higher concentration of anti-VHSV nucleocapsid serum antibodies in a well resulted in the absence of blue color and therefore higher percent inhibition of the mouse anti-VHSV nucleocapsid monoclonal binding to the VHSV antigen.

ROC analysis. A receiver operating characteristic (ROC) analysis was performed over a range of possible percent inhibition cutoff points for the ELISA (42). The thresholds were based on the percent inhibition values for the infected and uninfected fish.

Viral RNA analysis of serum by real-time RT-PCR. Two published real-time RT-PCR assays were used to detect viral RNA. At the Great Lakes Water Institute, University of Wisconsin (UW)-Milwaukee, RNA was extracted, and real-time PCR (21, 43) was performed on all yellow perch (Table 1). At UW-Madison, RNA was extracted, and real-time RT-PCR was performed (31) on the remaining fish. Any samples crossing the cycle threshold before cycle 40 were considered positive.

RESULTS

Prevalence of neutralizing antibodies in control serum by VN assay. The VN assay was modified from a previously described protocol (27). The results from the pilot studies (not shown) indicated that 24-h incubation of the virus and serum prior to inoculation onto the cells produced more significant neutralization of the virus versus a 30-min or 1-h incubation described in previously developed plaque neutralization test protocols for VHSV I and IVb (24, 44). The pilot studies also showed considerable toxicity at dilutions of 1:2 to 1:8. Thus, an initial dilution of 1:16 was used. Serum samples showing partial or complete protection were considered positive and were designated p1:16 (partial) or 1:16 (complete). If no protection was observed at 1:16, a serum sample was considered to be negative.

Neutralizing antibody titers were not detected in any of the serum samples from fish in the VHS-negative group ($n = 34$). Low VHSV-neutralizing titers were detected in 43% (12/28) of the fish from the VHS-positive group (Table 2), with titers ranging from p1:16 to 1:80. Thus, the VN assay had a specificity of 100% (95% confidence interval, 89.6% to 100%) and a sensitivity of 42.9% (95% confidence interval, 24.5% to 62.8%).

The addition of naive brown trout serum as complement was evaluated in our VN assay and was found to have no effect on neutralization (data not shown). A methylcellulose overlay was also evaluated for the isolation of plaques but was not necessary in reading the last serum dilution showing protection of the monolayer for determining the neutralizing antibody titer in the VN assay (data not shown). Three antibody-positive controls and one antibody-negative control were used to compare results with and without the addition of overlay, and no difference in antibody titer was observed.

Cross-reactivity of anti-VHSV nucleocapsid monoclonal antibody with SVCV. A Western blot under reduced conditions showed staining only with the nucleocapsid protein of VHSV using the anti-VHSV nucleocapsid monoclonal antibody, showing the specificity of the antibody to this protein (results not shown). No staining occurred with the SVCV lysate in a Western blot using the anti-VHSV nucleocapsid monoclonal antibody, indicating no cross-reactivity between our monoclonal detection antibody and SVCV. Specifically, these results show that there is no cross-recognition between the linear epitopes of the N proteins of VHSV and SVCV.

Analysis of anti-VHSV nucleocapsid monoclonal antibody in ELISA. A blocking ELISA is well suited for testing diagnostic samples from wildlife species because a secondary antibody is not required. At the time of assay development, no effective monoclonal antibody against the VHSV glycoprotein was available commercially. The anti-VHSV nucleocapsid monoclonal antibody used in this study was commercially available and effective. The anti-VHSV nucleocapsid monoclonal antibody has an advantage in that it detects persistent antibodies directed against the nucleocapsid. ELISA plates coated with intact viral particles revealed incomplete blocking. Treating the virus with MEGA-10 detergent prior to coating the plates was a critical step to allow for accurate identification of infected and noninfected fish. Presumably, this treatment reveals the target epitope of the nucleocapsid protein and allows the binding of the anti-nucleocapsid monoclonal antibody (45–47).

The efficacy of the anti-VHSV nucleocapsid monoclonal anti-

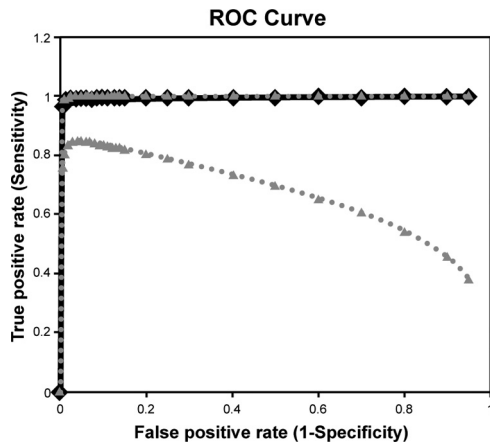


FIG 1 Receiver operating characteristic (ROC) curve of blocking ELISA using 1:2 diluted serum from VHSV-infected fish ($n = 28$) and uninfected fish ($n = 34$). The dashed lines indicate upper and lower 95% confidence intervals for the true-positive fraction, and the solid line indicates the curve for the true-positive rate.

body was evaluated by testing serum samples from the 34 uninfected and 28 previously infected fish. The serum samples were tested both undiluted and at a 1:2 dilution. The serum samples were tested at a 1:2 dilution to eliminate high background issues with hemolyzed serum.

Thirty of 34 serum samples (88.2%) from the VHS-negative group fish diluted 1:2 were negative by ELISA (Table 1). Twenty-seven of the 28 serum samples (96.4%) from the VHS-positive group fish diluted 1:2 were positive by ELISA (Table 2).

ROC analysis for ELISA. A receiver operating characteristic (ROC) curve was performed to derive the optimal percent inhibition threshold for detecting VHSV antibodies in fish serum (Fig. 1). Undiluted samples were considered positive at 35 to 100% inhibition and negative at <35% inhibition. The samples tested at a 1:2 dilution were considered positive at 25 to 100% inhibition and negative <25%, based on the ROC analysis. The area under the ROC curve was 0.994, confirming that the ELISA diagnostic performance characteristics under these thresholds were well correlated with the true status of each serum sample. These cutoff values demonstrated 88.2% specificity (95% confidence interval, 72.5% to 96.6%) and 96.4% sensitivity (95% confidence interval, 81.6% to 99.4%) with serum diluted 1:2. The percent inhibition values from uninfected fish ranged from 0.24% to 41.1% (average, 15.66%) and 7.94% to 95.99% (average, 49.21%) for previously infected fish. The positive predictive value of the ELISA for experimentally infected fish is 87.1% (95% confidence interval, 70.2% to 96.3%) and the negative predictive value is 96.8% (95% confidence interval, 83.2% to 99.5%) (Table 3 and 4).

Viral RNA detection by real-time RT-PCR. Serum samples

TABLE 3 Results of blocking ELISA^a

VHS infection status	No. of known positives ($n = 28$)	No. of known negatives ($n = 34$)
Positive	27	4
Negative	1	30

^a $n = 62$. The sensitivity is 96.4% and the specificity is 88.2%, both calculated from fish in the VHS-negative and VHS-positive groups.

TABLE 4 Results of VN assay^a

VHS infection status	No. of known positives ($n = 28$)	No. of known negatives ($n = 34$)
Positive	12	0
Negative	16	34

^a $n = 62$. The sensitivity is 42.9% and the specificity is 100%, both calculated from fish in the VHS-negative and VHS-positive groups.

were tested for VHSV by RT-PCR to determine if there was viral infection at the time blood was collected for fish exposed to VHSV and to determine if the inhibition of antibody binding was occurring in our tests due to antibodies being complexed with virus in the serum.

VHSV RNA was not detected by real-time RT-PCR in sera from uninfected fish. Sera from yellow perch H1-14, J1-13/J3-11, H4 A, H4 B, H4 C, and J4 A were positive, with threshold cycle (C_T) values ranging from 32.6 to 39.5 (Table 2). Serum from grass carp 2 was positive, with a C_T of 39.5, and that from Pacific herring 149 was positive, with a C_T of 38.9. All other sera from fish in the VHS-positive group tested negative for viral RNA.

DISCUSSION

We successfully developed a virus neutralization assay and a blocking ELISA to detect neutralizing and nonneutralizing antibodies against VHSV, respectively. The VN assay has the advantage of recognizing antibodies that likely confer protective immunity to VHSV and can indicate recent exposure to the virus (25). The blocking ELISA is valuable for identifying nonneutralizing anti-nucleocapsid antibodies, which may persist longer and therefore extend the opportunity to detect VHSV antibodies after initial virus exposure (25, 48). These assays complement viral detection methods by providing a means for determining the exposure histories of wild populations.

Previous studies have described the use of complement-dependent 50% plaque neutralization tests and indirect ELISAs to detect VHSV antibodies in trout (24, 25). These methods are reliable but not practical for screening large populations of fish from multiple species. There are currently no commercially available diagnostic tests in the United States for detecting antibodies to VHSV. Surveillance efforts by virus isolation are labor-intensive, must occur within narrow water temperature windows, and are costly. Although real-time PCR assays are available, these methods generally need to be performed in high-throughput laboratories for large sample sizes. Serological assays, such as our ELISA and VN assay, provide efficient and less costly methods for evaluating the VHSV exposure histories of samples from large wild fish populations or waterbodies.

Our VN assay is different from the traditional 50% plaque neutralization test (24, 44, 49) in that it is performed in a micro-neutralization format and the antibody titers are read as the last serum dilution showing complete protection of the cell monolayer from VHSV. Additionally, we determined that methylcellulose overlay is not necessary in our VN assay because plaques are not counted to determine the titers. Complement has been shown to enhance neutralization in 50% PNTs when applied to trout serum (23, 48, 50). However, neutralization was not enhanced by the addition of complement in our assay, which may indicate the presence of a different immune mechanism specific to trout. It should be noted that the reduced sensitivity observed in our VN

assay may be due to VHSV forming a complex with neutralizing antibodies in the serum, which reduces the availability of antibodies for binding to virus neutralization epitopes in the VN assay (45–48). However, this concern is obviated by using the nucleocapsid protein as our target, because antibodies with nucleocapsid affinity presumably do not complex with the intact viral particle, which underscores another value of the anti-nucleocapsid ELISA. It should be noted that four of the eight sera (50%) from our VHS-infected group tested positive for viral RNA but negative in the VN assay. This emphasizes the importance of utilizing parallel assays when testing the virus exposure history of fish.

Although previous experiments have determined that homologous strains of VHSV must be used for neutralization epitopes to be recognized (51, 52), serum samples from five Pacific herring hyperimmunized with VHSV type IVa were able to neutralize type IVb virus in the VN assay at low titers of p1:16 to 1:32. There are 21 amino acid differences between the type IVa and IVb glycoprotein sequences. None of these differences occur in two of the identified glycoprotein-neutralizing epitopes. This suggests there are shared neutralizing epitopes between types IVa and IVb (53, 54). Type IVa glycoprotein epitopes may be similar enough to those of type IVb to react in our VN assay. Indeed, a similar phenomenon was noted when Pacific herring vaccinated with the glycoprotein gene isolated from VHSV type Ia were protected from VHSV type IVb (55). Further investigation is needed to determine whether our VN assay may detect different antibody titers in herring exposed to virus that is homologous to that used in the VN assay. Additionally, although we demonstrate the ability to detect antibodies in hyperimmunized Pacific herring that likely experienced artificially high antibody titers, further investigations are needed to determine the sensitivities of these assays in wild Pacific herring or in those surviving more realistic VHSV exposure histories.

Our new blocking ELISA is a suitable nonlethal method for detecting exposure to VHSV. Considering the broad host range of VHSV type IVb (11), the advantage of a species-independent ELISA is significant for the surveillance of VHSV. The assay can measure the concentrations of antibodies directed against the nucleocapsid in any freshwater species since it does not require a secondary antibody. Furthermore, the ability of the monoclonal antibody to bind to a single viral epitope results in high specificity. We demonstrated by Western blotting that the nucleocapsid monoclonal antibody binding was specific to VHSV versus SVCV, another rhabdovirus that is present in Wisconsin. Previous studies showed a lack of cross-reaction between the antibody used herein and spring viremia of carp virus, infectious hematopoietic necrosis virus, pike fry rhabdovirus, or rhabdovirus anguilla (26, 56). According to the manufacturer of the antibody (Aquatic Diagnostics, Stirling, Scotland), no cross-reaction of the antibody occurs with nodavirus, infectious salmon anemia virus, koi herpesvirus, salmon alphavirus (1, 2, and 3), or *Piscirickettsia salmonis* infected cells. We were not able to test positive sera from transboundary VHSV strains (type I, II, and III); however, previous efforts indicate the anti-nucleocapsid monoclonal antibody detects anti-nucleocapsid antibodies against all strains of VHSV (26).

A feature crucial to the function of our ELISA is the treatment of the viral antigen with MEGA-10 detergent prior to coating the plates (47). Repeated trials showed that treatment of the virus with detergent allowed for better attachment of the nucleocapsid-specific antibody to the virus. This result is probably due to the ability

of the detergent to expose the nucleocapsid epitope and make it available for antibody binding.

The large difference in sensitivities between our blocking ELISA (96.4%) and VN assay (42.9%) may be attributed to the immune response kinetics at the time of serum collection. Studies have shown that neutralizing antibodies do not persist as long as nonneutralizing antibodies in trout (25, 48), and neutralizing antibody titers peak at 6 weeks postinfection in rainbow trout infected with VHSV I (24) and at 11 to 16 weeks in muskellunge infected with VHSV IVb (44). It was observed that the majority of VHSV-exposed fish with serum samples collected prior to 6 weeks postinfection had no or low neutralizing antibody titers. Those with low titers may have still been clearing virus while producing protective antibodies, indicated by the presence of viral RNA in the serum sample of a portion of our VHSV-exposed fish. Investigation into the kinetics of viral replication and the related immune response in multiple species are therefore important for further study.

A limitation of this study is the number of serum samples available from VHSV-uninfected and -infected fish. We used serum samples from 27 experimentally infected and one wild-caught fish that had VHSV-positive kidney and spleen tissues as tested by real-time RT-PCR for ELISA development. It is also important to note that our threshold for detecting VHSV antibodies by ELISA may require adjustment when evaluating various wild-caught species due to differing environments and susceptibility. In this light, we note that 4 of 34 serum samples diluted 1:2 from the VHS-negative group were positive on the blocking ELISA. There may be nonspecific reactions occurring that more extensive testing would help reconcile. For the purpose of this assay as a surveillance tool, it is more practical to keep a threshold at a level that maximizes sensitivity.

In summary, the blocking ELISA shows high sensitivity and acceptable specificity, whereas the VN assay has unacceptably low sensitivity but high specificity. When used in parallel, the VN assay and ELISAs correctly identified the VHSV exposure status of all known uninfected and infected fish. Our results highlight that the anti-VHSV nucleocapsid monoclonal antibody used in the blocking ELISA is a good indicator of past exposure to VHSV and may be a reliable time-independent and species-independent diagnostic test suitable for nonlethal surveillance of VHSV. Our nonlethal serological assays will be valuable for assessing VHSV exposure history and might reduce the extensive laboratory effort needed to screen fish for VHSV using virus isolation. Use of the VN assay, blocking ELISA, and virus isolation under actual surveillance conditions is needed to fully demonstrate the interplay between the assays. The collection of additional reference samples is required for continued assay validation to further assess the sensitivity and specificity and determine repeatability, robustness, and ruggedness. Our serological assays might supplement existing VHS surveillance protocols, which might have regulatory implications for fish movement between VHSV-positive and -negative locations in certain jurisdictions or geographic regions.

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