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Detection of viral hemorrhagic septicemia virus-IVb antibodies in sera of muskellunge *Esox masquinongy* using competitive ELISA

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ABSTRACT: A competitive enzyme-linked immunosorbent assay (cELISA) was developed for the detection of antibodies to viral hemorrhagic septicemia virus genotype IVb (VHSV-IVb) in fish sera. Assay conditions were standardized using known negative and positive muskellunge *Esox masquinongy*. A positive–negative threshold of 14.6% inhibition was established based on analysis of sera of 60 muskellunge with no previous exposure to VHSV-IVb. The cELISA was then used to investigate immune responses of wild muskellunge sampled from 5 water bodies in Michigan and Wisconsin, USA, between 2005 and 2012. Antibodies were detected in fish from Lake St. Clair, Michigan, and Lower Fox River/Green Bay, Wisconsin. Both water systems were considered enzootic for VHSV-IVb. Additionally, antibodies were detected in muskellunge from Thornapple Lake, a Michigan inland lake previously considered negative for VHSV-IVb based on virus isolation methods. Muskellunge populations from Lake Hudson, Michigan, and Butternut Lake, Wisconsin, lacked evidence of an immune response to VHSV-IVb. When results of the cELISA were compared to the 50% plaque neutralization test for several groups of fish, there was 78.4% agreement between the tests for antibody presence. The cELISA is a rapid and efficient test for the detection of binding antibodies to VHSV-IVb and will be a useful non-lethal tool for monitoring the spread of this serious pathogen.

KEY WORDS: Viral haemorrhagic septicaemia · VHSV · Serology · Surveillance · Immunoassay · Great Lakes · Muskellunge

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) (family *Rhabdoviridae*, genus *Novirhabdovirus*) is the causative agent of viral hemorrhagic septicemia (VHS). VHS is a World Organization for Animal Health (OIE) reportable disease of freshwater and marine fish. In the Great Lakes region of North America, a new sublineage of the virus, designated VHSV-IVb, has emerged within the last decade and has caused large-scale mortality events in wild freshwater fish populations (Elsayed et al. 2006, Groocock et al. 2007, Lumsden et al. 2007, Faisal et al. 2012). VHSV-IVb has since been isolated from at least 28 fish species (www.aphis.usda.gov/animal_health/animal_dis_spec/aquaculture/downloads/vhs_regulated_spp.pdf), and concerns exist regarding its continued spread to naïve fish populations and potential introduction into aquaculture systems. Ongoing VHSV-IVb surveillance efforts aim to determine virus distribution and delineate VHSV-IVb positive and negative zones in the Great Lakes basin. Testing for VHSV-IVb is com-

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monly conducted according to the international standard, which is virus isolation from fish tissues in cell culture followed by confirmation of the isolated virus by reverse-transcriptase polymerase chain reaction (OIE 2012, USFWS & AFS-FHS 2010). During and immediately following VHS outbreaks, VHSV can be readily isolated in this manner (OIE 2012, Schyth et al. 2012). Detection of the virus from clinically healthy fish (carriers) is more problematic and is dependent on many factors such as environmental temperature and time since exposure (OIE 2012).

In the absence of clinical VHS disease outbreaks, such as for surveillance purposes or monitoring enzootic populations, the use of antibody-based detection assays can increase the capacity to screen populations for exposure to VHSV (Hattenberger-Baudouy et al. 1995, LaPatra 1996, Millard & Faisal 2012b, Schyth et al. 2012). After the onset of antibody production, circulating antibodies have been detected in the range of months to years after VHS infection in sera of surviving fish (Olesen & Jørgensen 1986, Olesen et al. 1991, Enzmann & Konrad 1993, Millard & Faisal 2012a). Another benefit of serological testing is that it can be done non-lethally, an attribute that is especially ideal for the study of threatened, valuable, and long-lived species. It is important to consider that the presence of virus-specific antibody indicates seroconversion but does not necessarily indicate that fish ever developed clinical VHS disease and does not distinguish between subclinically infected and recovered fish.

Neutralizing antibodies are directed against viral surface glycoproteins (G) and are an important component of the protective immune response of rainbow trout *Oncorhynchus mykiss* to VHSV (Lorenzen et al. 1990, Béarzotti et al. 1995, Lorenzen & LaPatra 1999). This set of antibodies is measured by neutralization tests, such as the 50% plaque neutralization test (PNT), that rely on an exogenous source of fish complement (Doeson & Torchy 1979). Rainbow trout surviving infection with VHS also produce binding antibodies, and several indirect enzyme-linked immunosorbent assays (ELISA) have been developed for their detection (Jørgensen et al. 1991, Olesen et al. 1991, Fernandez-Alonso et al. 1998, Encinas et al. 2011). In some of these studies, binding antibodies were detected in a higher percentage of fish and lasted longer than did neutralizing antibodies. Compared to the PNT, ELISA was considered a more feasible test for surveillance studies given the capacity to screen a larger number of samples simultaneously, quicker test results, and reduced costs (Olesen et al. 1991, LaPatra 1996).

In this study, we developed a competitive ELISA (cELISA) to detect antibodies to VHSV-IVb and applied it to sera from wild muskellunge *Esox masquinongy* from systems where the virus is enzootic and systems where the virus has never been detected. Competitive ELISAs, such as the one developed by Dixon et al. (1994) for detection of spring viremia of carp virus (SVCV) antibodies, use a virus-specific antiserum to compete with fish sera for virus binding sites. As such, the cELISA can be used on multiple fish species without the need to develop specific anti-immunoglobulin reagents for each species tested as would be necessary for indirect ELISAs. This is a more feasible approach given the wide range of fish hosts for VHSV-IVb and the absence of commercially available antisera to immunoglobulins for many of these species. We focused on muskellunge for this initial study due to the known susceptibility of this species to VHSV-IVb (Kim & Faisal 2010) and the availability of sera from unexposed, experimentally infected, and naturally infected populations. Furthermore, Millard & Faisal (2012b) showed that muskellunge are good serological indicators for VHSV-IVb presence in an ecosystem based on the presence of neutralizing antibodies.

MATERIALS AND METHODS

Preparation of virus stock and purified virus

The MI03 index strain of VHSV-IVb (Elsayed et al. 2006) was used throughout this study. To produce purified virus for use as the cELISA coating antigen and for immunization of rabbits, VHSV-IVb was propagated in epithelioma papulosum cyprini (EPC) cells (Winton et al. 2010) in minimum essential medium with Earle's salts (EMEM; Life Technologies) supplemented with 10% tryptose phosphate broth (BD Biosciences), 2.0 mM L-glutamine (Life Technologies), and 12 mM sodium bicarbonate (Sigma-Aldrich). After cells had lifted, flasks were briefly frozen at –20°C, and cell debris was removed by centrifugation (5200 × *g*, 30 min, 4°C). Resulting supernatants were filtered using a Corning® bottle-top vacuum filter system and ultracentrifuged using a SW 32 Ti rotor (Beckman Coulter) at 80 000 × *g* (2.5 h, 4°C). Concentrated virus pellets were resuspended in TE buffer (20 mM Tris-HCl, pH 7.5, and 1 mM EDTA, pH 8.0) and stored at –80°C.

For purification, virus suspensions were overlaid on 10 to 60% discontinuous sucrose gradient columns and ultracentrifuged (80 000 × *g*, 2.5 h, 4°C).

The fraction containing the purified virus was used immediately for immunization of rabbits. For use as the cELISA coating antigen, this fraction was given a final wash by pelleting through TE buffer (80 000 × *g*, 150 min, 4°C). The resulting pellet of purified virus was resuspended in phosphate buffered saline (PBS: 0.138 M NaCl, 0.0027 M KCl; pH 7.4; Sigma-Aldrich), divided into aliquots, and stored at −80°C until use. The protein concentration of the final virus preparation was determined using a Qubit® Fluorometer with the Qubit® Protein Assay Kit (Life Technologies).

The virus used in experimental infections and the PNT was propagated in EPC cells as previously described (Millard & Faisal 2012b). The titers of the virus stocks were determined by a virus plaque assay on EPC cells. Cells were pretreated with a 7% solution of polyethylene glycol (PEG; 20 000 MW; JT Baker) (Batts & Winton 1989).

Production of polyclonal antibody to VHSV-IVb

Three New Zealand white rabbits were given a series of 2 immunizations of purified VHSV-IVb over a 5 wk period. Purified virus (0.5 ml, 10^{10-12} $TCID_{50}$) was emulsified 1:1 in Freund's complete adjuvant (Sigma-Aldrich) for the first immunization and in Freund's incomplete adjuvant (Sigma-Aldrich) for the second. The immunizations were administered subcutaneously over the dorsum (10 sites, 0.1 ml $site^{-1}$). Serum was collected from each rabbit prior to immunization (pre-immune sera) and 3 wk after the second immunization (hyperimmune serum). Sera were heat-treated at 45°C for 45 min, divided into aliquots, and stored at −80°C. Rabbits and fish were cared for in accordance with guidelines defined by Michigan State University's (MSU) Institutional Animal Care and Use Committee (AUF 07/07-123-00, 09/10-140-00, 02/10-013-00).

Competitive ELISA procedure and control sera

The immunoassay was initially established as an indirect ELISA system following general principles described by Voller et al. (1979) and Crowther (2000). A variety of types of plates, blocking and diluent reagents, and incubation times and temperatures were tested in preliminary trials, and titrations were carried out to determine several suitable reagent combinations. Purified virus (coating antigen) at a starting dilution of 16 µg ml^{-1} and rabbit hyperimmune serum to VHSV-IVb at a starting dilution of 1:2000 were tested in 2-fold serial dilutions. The anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich) was tested at dilutions of 1:1000, 1:2500, and 1:5000. Final concentrations for the competitive immunoassay were then optimized using 3 fish sera controls. The positive control sera were from 2 adult muskellunge collected from a VHSV-IVb-positive water body in Michigan that were positive for neutralizing antibodies by PNT (titers of 20 480 and 1280). The negative control consisted of a pooled serum sample from naïve hatchery-reared muskellunge from outside the Great Lakes basin that were negative by PNT. After standardization trials, fish sera controls were included on each assay plate and consisted of the high positive control (PNT titer of 20 480), a low positive control (1:80 dilution of the high positive), and the negative control serum.

Polystyrene microplates (96-well, Microlon®600 with chimney wells; Greiner Bio-One) were used as the solid phase for the cELISA. Plates were sealed for all incubations and washed 5 times with PBS containing 0.05% Tween 20 (PBS-T20; Sigma-Aldrich) in an automated microplate washer (BioTek, ELx405™) following each step unless otherwise stated. Briefly, assay plates were coated with 100 µl $well^{-1}$ of purified VHSV-IVb in PBS at a concentration of 1 µg ml^{-1} and incubated overnight (16–18 h) at 4°C in a humid chamber. Prior to running the assay, plates were washed, and unoccupied sites were blocked with the addition of 420 µl $well^{-1}$ of PBS containing 5% nonfat dried milk (PBS-5%NFDM; Sigma-Aldrich) for 1 h at 37°C. Fish control and test sera, diluted 1:10 in PBS-1%NFDM (hereafter referred to as sample diluent) and pretreated as described in the following paragraph, were then added to duplicate wells (100 µl $well^{-1}$). At this time, sample diluent alone was added to duplicate diluent control wells, and 12 wells reserved for the rabbit serum control. After incubating for 1 h at 25°C, rabbit hyperimmune serum to VHSV-IVb (1:512 000 in sample diluent, 100 µl $well^{-1}$) was added to rabbit serum control wells and all wells that had received fish serum during the previous step. Sample diluent alone was again added to diluent control wells, and plates were incubated for 1 h at 25°C. A commercial anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich), 1:1000 in sample diluent, was then added to all wells (100 µl $well^{-1}$), and the incubation period of 1 h at 25°C was repeated. Enzymatic development of the plates was started by the addition of substrate (0.4 mg ml^{-1} *o*-phenylenediamine in phosphate citrate buffer containing 3 mM hydrogen peroxide; 100 µl $well^{-1}$) and color development proceeded for 30 min at 25°C in the dark. Without washing, the reaction was stopped

with 3 M sulfuric acid. An absorbance microplate reader was used to read dual wavelength optical density at 405 and 490 nm ($OD_{405/490}$). The average $OD_{405/490}$ of the diluent control wells was subtracted from each well. Results were interpreted as the percent inhibition of the average $OD_{405/490}$ of the rabbit serum control. The following formula was used to calculate percent inhibition: [1 − (average $OD_{405/490}$ fish serum/average $OD_{405/490}$ rabbit serum control)] × 100. Negative inhibition values were treated as zeros in all analyses.

All fish control and test sera were heat-treated for 30 min at 45°C. The night prior to performing the assay, fish sera (22 µl each) were diluted 1:10 in sample diluent in 96-well dilution plates that had been pre-blocked with PBS-5%NFDM. Rabbit serum (1:1000) was also pre-incubated overnight prior to the assay in sample diluent.

Establishment of a positive–negative threshold for muskellunge

Muskellunge were obtained from Rathbun National Fish Hatchery (Iowa Department of Natural Resources, Moravia, Iowa), which is located outside the Great Lakes basin (hereafter referred to as naïve muskellunge). Naïve muskellunge were kept in a flow-through system at the MSU Research Containment Facility and fed fathead minnows *Pimephales promelas* purchased from Anderson Minnow Farm (Lonoke, Arkansas). Naïve muskellunge and minnows were certified to be free of VHSV and other reportable viruses. Blood was drawn from the caudal vein of 60 naïve muskellunge (average weight ~80 g) using a sterile syringe and needle. Blood samples were allowed to clot at 4°C overnight and then centrifuged (2500 × *g*, 10 min, 4°C). Sera were collected, divided into aliquots, and stored at −80°C. These sera were tested by the cELISA in order to determine a baseline level of inhibition present in sera of muskellunge that were not exposed to VHSV. The positive–negative threshold for the cELISA test was established by calculating the upper 95% confidence limit to the 95th quantile of the inhibition values for the naïve muskellunge. We chose to use this quantile approach for determining thresholds rather than the more common 'mean + 3 standard deviations (SD)' approach because its interpretation is not dependent on the underlying distribution of percent inhibition values of naïve fish sera. The approach is also more robust to decisions regarding how negative inhibitions are calculated and to potential anomalous observations. The quantiles and their upper 95% confidence limits were calculated in SAS 9.2 (SAS Institute 2010). Confidence limits for the quantiles were calculated using the bootstrapping approach of He & Hu (2002). In total, 5000 bootstrap samples were used to calculate the confidence limits.

Use of sera from fish surviving experimental VHSV-IVb infection to test assay

The cELISA was first tested using sera from 2 groups of muskellunge that were experimentally challenged with VHSV-IVb (MI03 strain) by immersion in previous studies. Fish (n = 60–90 per dose group) were challenged by immersion in aerated water containing 4×10^3 or 10^5 plaque-forming units (pfu) ml^{-1} VHSV-IVb (experimentally exposed fish) or an equal volume of tissue culture media (mock-challenged fish). Fish were sampled between 1 and 15 wk (11 and 165 degree days) post infection (PI). The first group of sera tested by the cELISA represents a subset of these samples and consisted of 27 sera from individual experimentally exposed fish and 16 mock-challenged fish (2 to 6 fish per time point). Further details of this trial can be found in Millard & Faisal (2012a).

The second group of sera consisted of 42 sera collected from 22 individual muskellunge (4 mo old, 13 g) that were survivors of 2 previous VHSV-IVb immersion trials (Kim & Faisal 2012). Fish (n = 234) were initially exposed to a low dose of the virus (1.4×10^3 pfu ml^{-1}), and survivors were re-challenged 22 wk (1700 degree days) later with doses ranging from 10 to 10^6 pfu ml^{-1}. The 22 fish that survived both challenges were later tagged with passive integrated transponder (PIT) tags, and serial blood samples were collected from the same individuals 67 and 79 wk (5200 and 6000 degree days) PI (45 and 57 wk post second exposure). Water temperature was maintained at approximately 11 ± 1°C for both studies.

Application of the assay to field-collected sera of muskellunge

A total of 200 blood samples were obtained from wild muskellunge sampled from 5 water bodies in Michigan (MI) and Wisconsin (WI) (see Table 2). Fish were sampled prior to or during spawning between 2005 and 2012. Most fish were of reproductively mature age. Four locations were within the Great Lakes basin and included 2 areas considered en-

zootic for VHSV-IVb (Lake St. Clair, MI, and Lower Fox River/Green Bay, WI) and 2 inland lakes (Lake Hudson and Thornapple Lake, MI) considered negative for VHSV-IVb based on past viral testing. The fifth water body (Butternut Lake, WI) was a VHSV-negative inland lake located outside the Great Lakes basin. Muskellunge from Lake St. Clair were sampled in spring 2010 and spring 2011 near Anchor Bay and the Detroit River (Fig. 1). Lower Fox River muskellunge were collected in spring 2010, 2011, and 2012 near the mouth of the Fox River at Green Bay. Lower Fox River muskellunge are primarily residents of Green Bay waters of Lake Michigan except during spawning. The last isolations of VHSV-IVb from fish in Lake St. Clair and Green Bay (and connected waters) were in spring 2009. As part of routine monitoring efforts in the states of MI and WI, sera were collected from muskellunge from Butternut Lake in spring 2005 and 2007 and from Lake Hudson and Thornapple Lake in spring 2008 and 2010.

No fish were euthanized for the purposes of this study. Blood samples were collected non-lethally, or from fish euthanized for other purposes (health inspections or VHSV-IVb surveillance for state regulatory agencies). All samples from fish tested in this study were negative for VHSV and other viruses using virus isolation in cell culture as described by Millard & Faisal (2012b), Faisal et al. (2012), and the WI Department of Natural Resources (unpublished). Samples tested by virus isolation included internal tissues from all Butternut Lake fish (kidney, spleen) and most Lake St. Clair fish (kidney, spleen, heart), and non-lethal samples (reproductive fluids and/or sera) from all fish from Lakes St. Clair and Hudson, Lower Fox River, and Thornapple Lake.

Comparison of cELISA and PNT results

Results obtained by cELISA and PNT were compared for several groups of muskellunge to assess the agreement between the 2 methods. The first group consisted of fish from the first experimental immersion trial (n = 27, sampled between 1 and 15 wk PI). The second group consisted of the wild

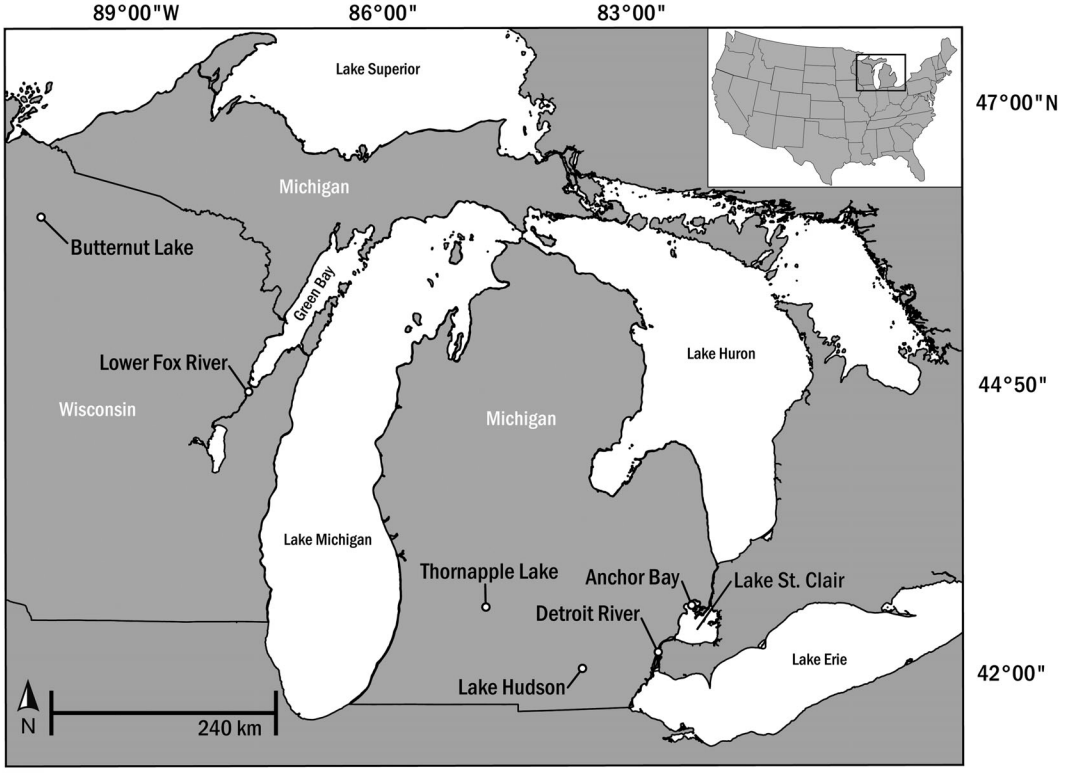

Fig. 1. *Esox masquinongy.* Michigan (MI) and Wisconsin (WI), USA, showing sites where sera were collected from muskellunge during health surveys of wild fish, 2005 to 2012. Fish sampled from the Anchor Bay and Detroit River sites are collectively referred to as Lake St. Clair, MI, muskellunge in this study. Muskellunge sampled from the Lower Fox River, WI, are primarily residents of Green Bay waters of Lake Michigan except during spawning

muskellunge from Lake St. Clair (n = 38) and Lower Fox River (n = 23). Muskellunge from Lower Fox River (all years) and muskellunge from Lake St. Clair (2011) were tested by PNT for this study. PNT results of remaining sera as well as details of the assay are as reported by Millard & Faisal (2012a,b). Briefly, 2-fold dilutions of heat-treated test sera and positive and negative controls were combined with an equal volume of pre-titrated VHSV-IVb. Mixtures were incubated for 30 min at 18°C. Unheated sera from naïve lake trout *Salvelinus namaycush* were then added as a source of complement and the incubation was repeated. Samples were plaque assayed on EPC monolayers and fixed and stained with 0.5% crystal violet in 50% formalin after 6 d. The neutralization titer of each test serum was defined as being the reciprocal of the highest serum dilution that prevents the formation of 50% of the viral plaques in relation to the negative control sera. Cohen's kappa was used to assess agreement of the assays for classifying muskellunge as positive or negative for antibodies based on the respective thresholds (PNT ≥160, cELISA ≥14.6%). Spearman rank correlation was used to assess correlation between PNT and cELISA titers.

RESULTS

cELISA development

The individual rabbit hyperimmune serum that was selected for use in the assay neutralized VHSV-IVb *in vitro* and had a strong reactivity to the virus in preliminary indirect ELISA trials. Pre-immune serum from this rabbit was non-neutralizing and did not bind to purified virus in the ELISA. In preliminary trials, we determined that blocking and diluent reagents containing nonfat milk reduced background levels better than did reagents with bovine serum albumin or goat serum. Incubation periods were carried out at 25°C since temperature could be maintained consistently in an incubator compared to room temperature. Sufficient washing after each step with PBS-T20, as opposed to PBS alone, was critical for inhibition detection and assay reproducibility.

In a series of cELISAs, we determined that inhibition was best detected at any given dilution of fish control sera when (1) the virus was coated to plates under non-saturating conditions, (2) the maximum absorbance of the rabbit serum control wells was approximately 1.0 ± 0.2 OD, and (3) the conjugate was used at higher compared to lower concentrations. At the minimum conjugate dilution tested (1:1000), negligible background was present in diluent control wells and the reagent was still economical, so this dilution was used for subsequent trials. The final concentrations of purified virus and rabbit hyperimmune serum for use in the cELISA were determined by titrations in the presence of 1:10 dilutions of negative and positive fish sera controls. The optimal concentration of virus was determined to be 1.0 µg ml^{-1} VHSV-IVb (0.1 µg $well^{-1}$), and the optimal dilution of rabbit hyperimmune serum was 1:512 000. Using these conditions, rabbit serum control wells resulted in an OD of approximately 1.0 and the difference between percent inhibitions of positive and negative fish sera controls was maximized. Positive fish sera controls effectively inhibited rabbit serum from binding and resulted in inhibition of color development by 86% and 84% compared to rabbit control wells. The negative control serum resulted in only 3 to 6% inhibition. Using these concentrations, results indicated that the cELISA was capable of making a clear distinction between muskellunge sera with and without antibodies to VHSV-IVb. These reagent concentrations were used for all subsequent assays.

Several treatments of the fish control sera were tested during preliminary trials. Heat-treatment of the serum resulted in lower non-specific binding of negative fish sera. Incubating overnight in diluent containing 1%NFDM also reduced non-specific binding. Using the final cELISA protocol and reagent concentrations, a small trial was done to determine whether fish sera should be screened at a dilution higher than 1:10. A panel of fish sera with and without VHSV-IVb antibodies was tested at dilutions of 1:10, 1:20, 1:40, and 1:80 (data not shown). At a serum dilution of 1:10, optimal positive:negative ratios for fish sera were achieved, so all test sera for this study were screened at the single, fixed dilution of 1:10. This method of testing was selected over an end-point dilution technique so that large numbers of sera could be screened at once.

Establishment of a positive–negative threshold for muskellunge

The distribution of inhibition values of sera from naïve fish used to establish the positive–negative threshold is shown in Fig. 2. Of the 60 naïve muskellunge, sera from 50 fish (83.3%) exhibited less than 5% inhibition of the rabbit serum, and 58 fish (96.7%) showed less than 10% inhibition. The observed

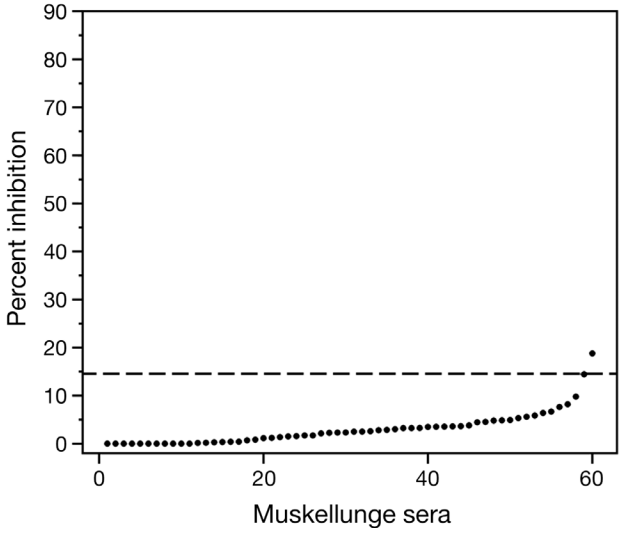

Fig. 2. *Esox masquinongy.* Distribution of cELISA percent inhibition values from sera of naïve muskellunge (n = 60) that were used to establish a positive–negative threshold of 14.6% inhibition (dashed line)

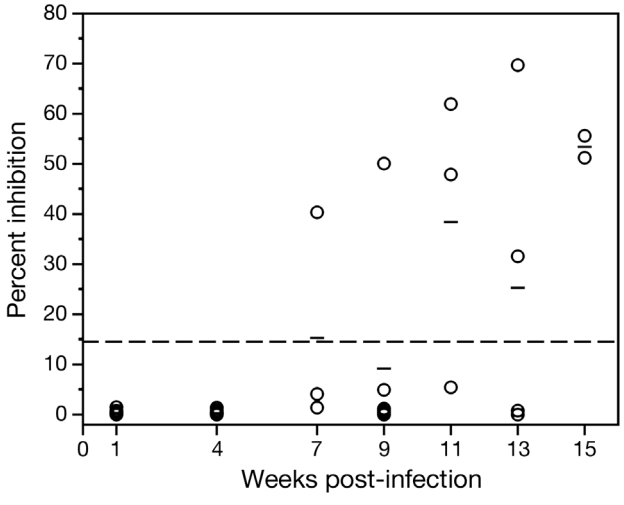

Fig. 3. *Esox masquinongy.* Percent inhibition values by cELISA of muskellunge (n = 27) sampled between 1 and 15 wk after immersion challenge with VHSV-IVb. Samples with percent inhibition values ≥14.6 (dashed line) are positive for antibodies to VHSV-IVb. Horizontal lines represent the mean percent inhibition

range of the inhibition values in naïve muskellunge was 0 to 18.8% (mean, SD = 3.0%, 3.5%). A positive–negative threshold of 14.6% inhibition was obtained using the upper 95% confidence limit to the 95th quantile of the inhibition values. For the purpose of this study, inhibition values ≥14.6% were considered positive for VHSV-IVb antibodies. Using the traditional 'mean + 3 SD' approach would have resulted in a similar threshold (13.5%).

Table 1. *Esox masquinongy.* Percent inhibitions of muskellunge testing positive (≥14.6% inhibition) for VHSV-IVb antibodies by cELISA at ≥1 time point sampled. Fish were challenged at 0 and 22 wk with VHSV-IVb by immersion, and blood was sampled from 22 survivors 67 and 79 wk post infection (45 and 57 wk post second exposure)

Fish no.	Percent inhibition	
	67 wk	79 wk
1	14.7	17.9
2	16.3	18.2
3	23.4	29.2
4	33.2	33.4
5	20.6	23.6
6	31.5	29.8
7	9.7	21.5
8	28.4	46.1
9	11.2	25.1
10	11.4	16.3
No. seropositive (%)	7 (31.8%)	10 (50.0%)
No. fish total	22	20[a]
Mean positive (SD)	24.0 (7.3)	26.1 (9.0)

[a]No blood could be collected from 2 fish

Use of sera from fish surviving experimental VHSV-IVb infection to test the cELISA

Inhibition values from fish that survived the single VHSV-IVb challenge are shown in Fig. 3. Inhibition was first detected 7 wk PI (33.3% of fish). The highest proportions of cELISA positive fish were detected 11 wk (66.7%) and 15 wk (100%) PI. Of the 18 fish sampled after the onset of antibody production (between 7 and 15 wk PI), 8 fish (44.4%) blocked the rabbit hyperimmune serum from binding to VHSV-IVb during the assay. Inhibition values of seropositive fish ranged from 31.6 to 69.7% (mean, SD = 51.0%, 11.9%). The range in inhibition for the 16 mock-challenged fish in contrast was 0.0 to 3.1% (1.3%, 1.4%) (data not shown).

Of the 22 muskellunge that survived 2 VHSV-IVb exposures, inhibition levels above the threshold were detected in sera of 7 fish (31.8%) sampled 67 wk PI (45 wk post second exposure) and in 10 fish sampled 79 wk PI (57 wk post second exposure; Table 1). Inhibition values of seropositive fish ranged from 14.7 to 46.1% (25.2%, 8.2%).

Application of the assay to field-collected sera of muskellunge

Inhibition values of all wild fish are shown in Fig. 4 by location and year, and data are summarized in Table 2. Seroprevalence is given as apparent (versus

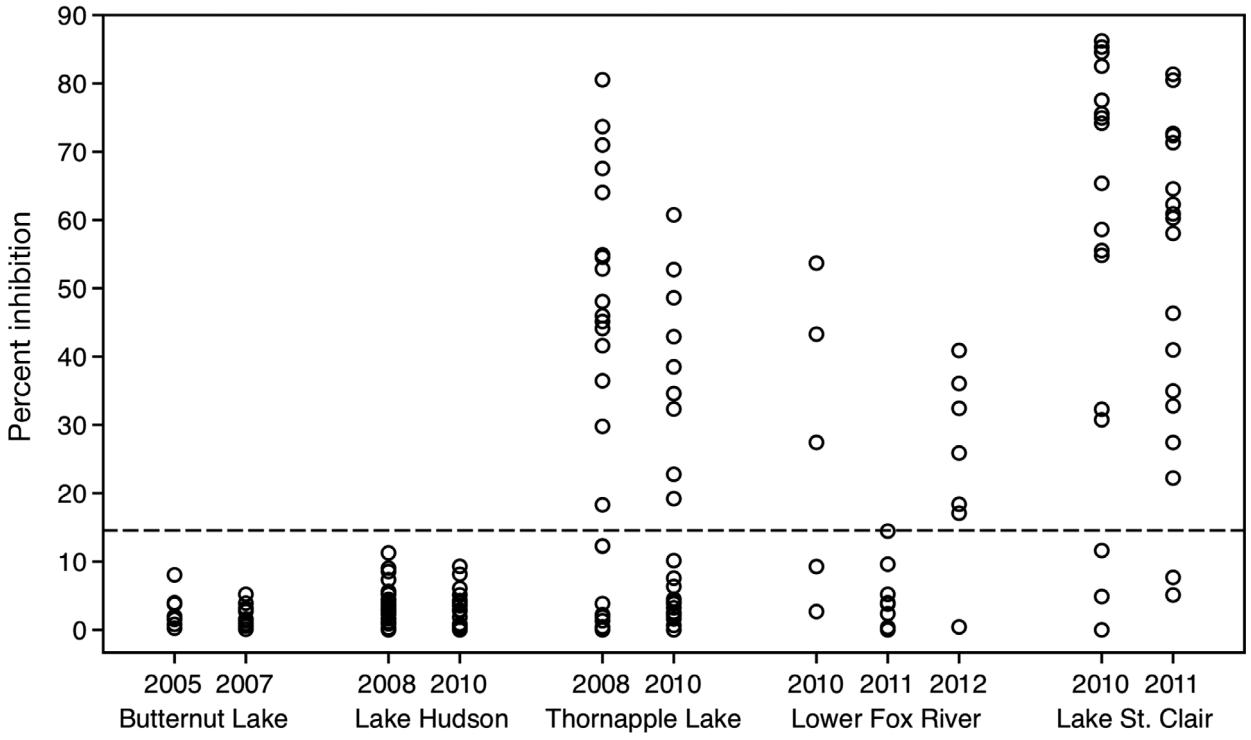

Fig. 4. *Esox masquinongy.* Distribution of percent inhibition values by cELISA of sera from 200 wild muskellunge by sampling location and year. Samples with percent inhibition values ≥14.6 (dashed line) are positive for antibodies to VHSV-IVb

Table 2. *Esox masquinongy.* Competitive ELISA results for 200 free-ranging muskellunge shown by location and sampling event. Fish were sampled between 2005 and 2012 from water bodies in Michigan (MI) and Wisconsin (WI), USA. Sera with percent inhibition values ≥14.6 by cELISA are positive for antibodies to VHSV-IVb. Tissues and/or non-lethal samples (ovarian fluid, sera) from all fish were negative for VHSV by cell culture isolation. A positive VHSV-IVb status indicates that the virus had been isolated from fish from that water system prior to the first sampling event. Confidence intervals (CI) were calculated using R (R Development Core Team 2012)

Location	VHSV-IVb status	Sampling event	No. of fish	% cELISA positive (95% CI)	Percent inhibition		
					Mean positive (SD)	Overall mean (SD)	Range
Butternut Lake, WI	Negative	2005, Apr 2	8	0	–	2.8 (2.5)	0.3–8.1
		2007, Apr 24	10	0	–	2.2 (1.6)	0.1–5.2
Lake Hudson, MI	Negative	2008, Apr 11	30	0	–	3.3 (2.9)	0.0–11.3
		2010, Apr 7	30	0	–	2.4 (2.9)	0.0–9.8
Thornapple Lake, MI	Negative	2008, Apr 11	30	53.3 (35.8–70.1)	51.8 (16.7)	28.8 (27.9)	0.0–80.5
		2010, Mar 31	30	30.0 (16.4–48.3)	39.2 (13.6)	13.6 (18.6)	0.0–60.8
Lower Fox River, WI	Positive	2010, Apr 28	5	60.0 (20.4–90.0)	41.5 (13.2)	27.3 (21.7)	2.7–53.7
		2011, May 10	12	0	–	4.0 (4.3)	0.0–14.5
		2012, May 1	7	85.7 (41.9–98.0)	28.5 (9.7)	24.5 (13.8)	0.4–40.9
Lake St. Clair, MI	Positive	2010, May 5–19	18	83.3 (59.1–94.5)	68.2 (18.3)	57.7 (29.3)	0.0–86.2
		2011, May 26–Jun 3	20	90.0 (67.6–97.5)	58.9 (20.3)	53.6 (25.1)	5.1–86.8

true) seroprevalence by definition since sensitivity and specificity of this assay have not been determined. Of the 38 muskellunge collected from Lake St. Clair, 33 fish (86.8%) had antibodies to VHSV-IVb based on the threshold of 14.6% inhibition. Seroprevalence was 83.3% in 2010 and 90.0% in 2011. Inhibition values for all Lake St. Clair muskellunge ranged from 0.0 to 86.8% (55.6%, 26.9%). Inhibition values of seropositive fish from Lake St. Clair ranged from 22.2 to 86.8% (63.1%, 19.7%). Mean positive antibody titer was slightly greater in May 2010 (68.2, 18.3) compared to May 2011 (58.9, 20.3).

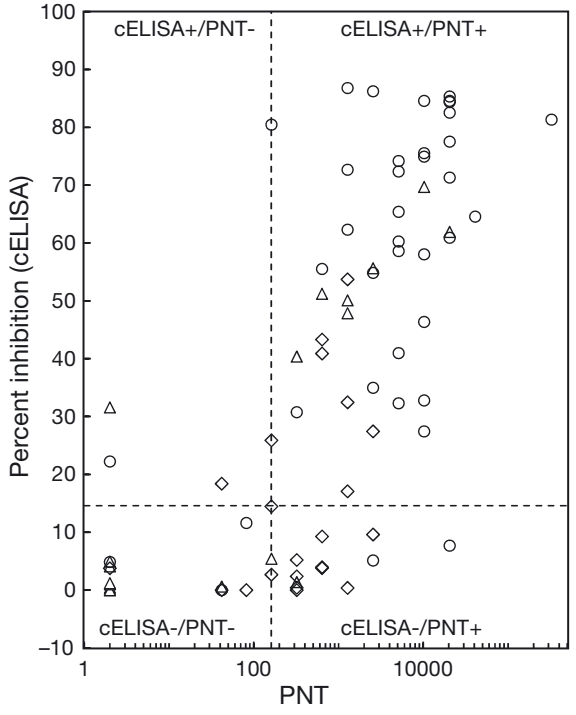

Fig. 5. *Esox masquinongy*. Comparison between cELISA and 50% plaque neutralization test (PNT) using sera from 38 wild muskellunge from Lake St. Clair, Michigan (○), 23 muskellunge from Lower Fox River, Wisconsin (◇), and 27 experimentally exposed muskellunge sampled 1 to 15 wk post exposure (△). Titers ≥14.6% inhibition (cELISA) and ≥160 (PNT) are considered positive for VHSV-IVb antibodies. In order to display all titers, PNT titers were arbitrarily transformed by adding 2 and resulting values plotted on a logarithmic scale

Of the 24 muskellunge from Lower Fox River tested by cELISA, 9 fish (37.5%) had antibodies to VHSV-IVb. Antibodies were detected in 60.0% of fish in April 2010 and 85.7% of fish in May 2012. No antibodies were detected in fish sampled in May 2011. Inhibition values of all seropositive fish ranged from 17.1 to 53.7% (32.8%, 12.0%). The mean positive antibody titer was higher in fish sampled in April 2010 (41.5%, 13.2%) compared to May 2012 (28.5%, 9.7%).

All 78 muskellunge collected from Butternut Lake (n = 18) and Lake Hudson (n = 60) were negative for VHSV-IVb antibodies. For Butternut Lake fish, inhibition values ranged from 0.1 to 8.1% (2.5%, 2.0%). For Lake Hudson fish, inhibition values ranged from 0.0 to 11.3% (2.9%, 2.9%).

Thornapple Lake, like Butternut Lake and Lake Hudson, was considered to be VHSV-free based on past viral testing. Muskellunge in Thornapple Lake, however, showed evidence of an immune response against VHSV-IVb. Of the 60 fish collected in 2008 and 2010, 25 fish (41.7%) had inhibition levels greater than the threshold. Seroprevalence and mean positive antibody titers were greater for fish sampled in April 2008 compared to 2010. In 2008, 16 of 30 muskellunge (53.3%) were seropositive. Inhibition values for cELISA positive fish in 2008 ranged from 18.3 to 80.5% (51.8%, 16.7%). In 2010, 9 of 30 muskellunge (30.0%) had antibodies to VHSV-IVb. Inhibition values of seropositive fish in 2010 ranged from 19.2 to 60.8% (39.2%, 13.6%). Inhibition values for all Thornapple muskellunge ranged from 0.0 to 80.5% (21.2%, 24.7%).

Comparison of cELISA to PNT results

Results by cELISA and PNT agreed in classifying fish as positive or negative for antibodies to VHSV-IVb for the majority of sera tested (Fig. 5). Overall, results were in agreement for 69 of 88 fish tested by both assays (78.4%, Cohen's kappa coefficient = 0.55). For the experimentally exposed fish alone, 29.6% were positive by cELISA and 33.3% were positive by PNT. Agreement between the tests was 88.9% (24 of 27 fish; Cohen's kappa coefficient = 0.74). Of the observations that were in agreement, 7 of 24 fish (29.2%) were positive by both assays and 17 of 24 fish (70.8%) were negative by both assays. For the wild muskellunge from Lake St. Clair and Lower Fox River, 41 fish (67.2%) were positive by cELISA and 53 fish (86.9%) were positive by PNT. Agreement between the tests for wild muskellunge was 73.8% (45 of 61; Cohen's kappa coefficient = 0.3). For the samples that agreed, 39 of 45 fish (86.7%) were positive by both assays and 6 of 45 fish (13.3%) were negative by both assays. Several sera from experimentally challenged and wild muskellunge were positive by only 1 assay. Of results that disagreed, 16 fish were PNT positive/cELISA negative and 3 fish were PNT negative/cELISA positive. Over half (56.3%) of the sera that were PNT positive/cELISA negative were from muskellunge sampled from the Lower Fox River in May 2011. There was some positive correlation between neutralizing titers and cELISA percent inhibition values. The Spearman correlation coefficients were 0.80 ($p < 0.0001$) and 0.6 ($p < 0.0001$) for experimentally exposed and wild fish, respectively.

DISCUSSION

In the current study, we developed a cELISA for the detection of antibodies in sera of fish previously exposed to VHSV-IVb under experimental and natu-

ral (field) conditions. Competition and inhibition ELISAs are used to quantify a substance (in our case, serum antibodies to VHSV-IVb) by measuring its ability to interfere with an established pre-titrated direct or indirect ELISA system (Crowther 2000). In our assay, microplates coated with purified VHSV-IVb were used as the solid phase. Fish test sera and rabbit serum (as a source of polyclonal antibody to VHSV-IVb) were added sequentially, and bound rabbit immunoglobulin was then detected with a commercially available enzyme-labeled conjugate. When optimizing reagent concentrations, we found that inhibition of the rabbit antiserum was most apparent when the purified virus was used at a non-saturating concentration (1.0 µg ml^{-1}) and when the maximum absorbance in the absence of inhibition was approximately 1.0 OD. In wells that previously received fish serum containing antibodies to VHSV-IVb, the polyclonal antibody was effectively outcompeted (or blocked) from binding, thus inhibiting enzymatic color change in those wells. This variation of the competitive assay is sometimes referred to as a blocking ELISA (Crowther 2000, Jordan 2005). Expressed on a scale of 0 to 100% inhibition of the competing antibody, results are proportional to antibody activity in the test sample (Wright et al. 1993). The presence of immunoglobulins in several sera that produced inhibition was confirmed in preliminary trials using an indirect ELISA system with a monoclonal antibody produced against muskellunge immunoglobulin M (S. L. Kaattari et al. unpubl.). However, the main reason for developing a competitive ELISA was to provide a test that could be used on multiple Great Lakes fish species, even on the same plate, without the need for developing species-specific immunoglobulin reagents as would be required for indirect ELISAs. Application of the assay for testing antibodies in other species was beyond the scope of this initial study.

Fish sera were heat-treated (Dixon et al. 1994, Kibenge et al. 2002) and pre-incubated with nonfat milk, as suggested by Kim et al. (2007), and purified virus was used to coat plates to minimize the possibility for false positive reactions. These measures were undertaken since some untreated sera from normal fish can exhibit high levels of non-specific binding (Olesen et al. 1991, Kibenge et al. 2002, Kim et al. 2007). Using the aforementioned conditions, sera from 60 juvenile muskellunge reared in a hatchery setting and considered to be naïve to VHSV-IVb were tested by the cELISA. Most sera produced inhibition values less than 5%, indicating only a low level of non-specific binding. With these data, we established a threshold (14.6% inhibition) above which inhibition would be unlikely to be due to non-specific binding. For the purposes of this study, we considered sera with inhibition ≥14.6% to be positive for antibodies to VHSV-IVb. While the threshold provided a means for us to interpret our results, we recognize that serological thresholds are often dynamic and can be biased based on extrinsic (e.g. diet, presence of other pathogens) and intrinsic factors (e.g. species, age, sex) of the host, as well as by laboratory testing methods. A large number of sera of known antibody-negative and antibody-positive status would be needed in order to refine the threshold in the future; however, this is difficult in the absence of a true gold standard reference test for determining VHSV-IVb seropositivity.

Using sera from experimental trials, we determined that the cELISA could detect antibodies to VHSV-IVb in some fish that had been previously exposed to the virus. The threshold provided a clear distinction between seropositive and seronegative fish. Antibodies were first detected in fish sampled 7 wk post immersion exposure, and the highest percentage of seropositive fish was detected later, between 11 and 15 wk PI. Low levels of inhibition could still be distinguished in sera from another group of muskellunge that was experimentally challenged approximately 1 yr prior to sampling. It is important to consider that not all fish exposed to the virus by immersion necessarily become infected, and furthermore, not all infected fish necessarily seroconvert.

When the cELISA was applied to wild populations of muskellunge, VHSV-IVb antibodies were detected in 3 of 5 populations tested. Muskellunge from 2 populations (Butternut Lake, WI, and Lake Hudson, MI) were negative for antibodies based on the threshold. Muskellunge from both locations were expected to be seronegative, as VHSV has never been detected from fish from these locations. Antibodies were detected in 60 to 90% of muskellunge from Lower Fox River sampled in 2010 and 2012 and in 80 to 90% of fish from Lake St. Clair sampled in 2010 and 2011. These 2 locations were considered enzootic for VHSV-IVb. The virus has been present in Lake St. Clair since at least 2003 and in Green Bay and its connected waters since 2007 (reviewed by Faisal et al. 2012). Prior to sampling, VHSV-IVb was last confirmed from fish in these systems in the spring and early summer 2009. Seroconversion may be due to recent exposure(s), indicating the persistence of the virus in these populations. Alternatively, given the longevity of muskellunge, it is possible that fish

were survivors from the last known occurrence of VHSV in these areas (2009). At the time of sampling, fish may have been fully recovered or may have been asymptomatic carriers of the virus, perhaps in tissues not sampled or at levels below detection limits of cell culture isolation. The high seroprevalence detected in fish from Lake St. Clair and Lower Fox River likely indicates that some immunity has been established to VHSV-IVb in these populations. While the association between virus-neutralizing antibodies and protective immunity against fish rhabdoviruses is well established (reviewed by Lorenzen & LaPatra 1999, Purcell et al. 2012), less is known about the role of binding antibodies. Binding antibodies in general function to opsonize pathogens for removal by phagocytes.

Antibodies were also detected in wild muskellunge from Thornapple Lake. Approximately 50% of muskellunge were seropositive in 2008 and 30% of fish were seropositive in 2010. Thornapple Lake is an inland lake in Michigan that was not considered to be positive for VHSV-IVb based on previous testing by virus isolation of reproductive fluids and/or sera from 60 muskellunge from this population collected yearly since 2006. The finding that fish in Thornapple Lake had prior exposure to VHSV-IVb was not surprising given this lake's high angler use and proximity to VHSV-IVb-positive regions of the Lake Michigan watershed. Although the presence of cross-reactive antibodies could be an alternative explanation for this finding, this is unlikely, given that no other fish rhabdoviruses are known to occur in Michigan. Our results suggest that the use of serological tests for detecting VHSV-IVb antibodies in fish sera can increase the likelihood of detecting, indirectly, the presence of the virus in a population (or water system) in the absence of clinical disease outbreaks. Given the longevity of antibodies compared to the time in which the virus can be isolated, the likelihood of detecting VHSV exposure based on antibodies could be expected to be higher than the likelihood of detecting the virus itself on most sampling occasions, the exception being when fish are sampled during outbreaks of clinical disease; this was shown recently for cultured rainbow trout naturally infected with VHSV-I in Denmark (Schyth et al. 2012). This has especially important implications when clinically healthy fish are being tested, as is done for surveillance purposes. In fish that have high levels of circulating antibodies, virus may be cleared even more rapidly from tissues following infection, further decreasing the time frame in which VHSV could be isolated.

In the absence of providing preliminary estimates of diagnostic sensitivity and specificity, due to the fact that we do not have a gold standard reference test for determining true antibody status, Cohen's kappa was used to compare agreement with the PNT. An overall kappa value of 0.55 indicated a moderate level of agreement beyond chance in the assays' ability to differentiate seropositive and seronegative muskellunge (Landis & Koch 1977). Agreement was not necessarily expected due to differences in the types of antibodies each assay measures, and this has been discussed in the context of several fish rhabdoviruses (Olesen et al. 1991, Ristow et al. 1993, Smail & Snow 2011). While the PNT measures antibodies directed against neutralizing epitopes of viral glycoproteins, immunoassays measure binding antibodies that could react with non-neutralizing G protein epitopes, as well as any other exposed virus proteins (e.g. nucleoprotein, matrix protein). Given the greater diversity of antibodies theoretically detectable by the cELISA, it is interesting that the majority of discrepant results in this study came from sera that were actually PNT positive/cELISA negative; this requires further study. Binding antibodies have been found to persist longer than neutralizing antibodies in sera of rainbow trout following experimental infection with VHSV-I (Olesen et al. 1991, Encinas et al. 2011). This was not evident in the current study for muskellunge following experimental infection with VHSV-IVb for the time period tested (1–15 wk PI), but it is possible that an extended sampling period or sample size would have revealed less agreement and correlation. Less agreement and correlation was detected for wild fish, and this could reflect differences in sampling time in relation to exposure time and number of exposures. Further studies are needed to characterize the types of antibodies detected by the cELISA and the kinetics of production and duration of neutralizing and binding antibodies against VHSV-IVb in Great Lakes fish.

The cELISA has the potential to be a useful, non-lethal research and/or surveillance tool to detect fish that have survived infection with VHSV-IVb. Compared to the PNT, the cELISA assay has a faster turn-around time for results and is easier to perform and less costly in terms of both technician time and materials. We suspect the further use of the cELISA as a supplement to VHSV-IVb surveillance efforts in the Great Lakes will reveal a wider distribution of the virus than is currently known.

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