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ARTICLE

Assessment of a Serologic Diagnostic Test and Kinetics of Antibody Development in Northern Pike Experimentally Infected with Viral Hemorrhagic Septicemia Virus

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Abstract
Viral hemorrhagic septicemia virus (VHSV) is an ongoing cause of disease and mortality in freshwater fishes across the Great Lakes region of the Midwestern United States. Antibody detection assays such as enzyme-linked immunosorbent assay (ELISA) are nonlethal serological methods that can have significantly shorter turnaround times than the current validated viral detection diagnostic methodology for VHSV: cell culture with confirmation by polymerase chain reaction (PCR). This study evaluated an ELISA that detects nonneutralizing antinucleocapsid antibodies to VHSV in Northern Pike *Esox lucius*. Juvenile Northern Pike were experimentally infected with VHSV by intraperitoneal injection. The infected fish were monitored for 12 weeks for signs of disease, and weekly serum samples were obtained. An analysis of the survival data showed that mortality occurred significantly more quickly in inoculated fish than in control fish. Fish that were infected by injection showed a significant increase in antibody response by 2 weeks postinfection. However, variation in the rate and pattern of antibody response among the infected fish was high at any given point. The optimum window for detecting antibodies in Northern Pike is 2–12 weeks postinfection, which generally follows the median time to appearance of clinical signs (21 d postinfection). The receiver-operating characteristic curve analysis showed the ELISA to have a sensitivity of 80.5% and a specificity of 63.2% in Northern Pike, but these values can be adjusted by choosing different percent inhibition cutoffs, which may facilitate the use of the test for specific management goals. The results of this study offer insights into the disease progression and immune kinetics of VHSV, including interindividual variation, which will aid in the management of this economically important virus.

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Viral hemorrhagic septicemia virus (VHSV) is a rhabdovirus Rhabdoviridae: *Novirhabdovirus* that is affecting a diversity of fish species worldwide (Wolf 1988; Kim and Faisal 2011; Millard and Faisal 2012; Millard et al. 2014; Wilson-Rothering et al. 2014, 2015). A freshwater strain, Great Lakes VHSV-IVb was first detected in Muskellunge *Esox masquinongy* in Lake Saint Clair, Michigan, in 2003, and has since been associated with large-scale mortality in 31 freshwater fish species (Kim and Faisal 2010a, 2010b; Faisal et al. 2012; Olson et al. 2013). Because VHSV is a reportable pathogen according to many state and federal agencies, fish are tested for VHSV as a part of routine fish health inspections and disease surveillance efforts. The current and most commonly used approved presumptive diagnostic testing method for VHSV is virus isolation in cell culture with a confirmatory polymerase chain reaction (PCR); however, other options for confirmatory diagnosis are available (Batts and Winton 2014). Viral cell culture requires tissue samples (kidney, spleen, heart, or brain) or ovarian fluids to be cultured on susceptible cell lines (e.g., *Epithelioma papulosum cyprini* or endothelial progenitor cells) for 14 d (although a positive result may appear sooner), followed by a 14-d blind passage prior to confirmatory PCR testing (Batts and Winton 2014; OIE 2018). This virus detection method involves lethal sampling, has up to a 4-week turnaround time for results, and detects current infection but not prior exposure. Rapid antibody detection methods, such as serologic methods, are nonlethal and could reduce turnaround time significantly. In addition, while both virus detection and serologic methods are useful for disease detection, serologic methods can improve surveillance and provide a better indication of the true prevalence of infection within a population (OIE 2018).

In the past decade, enzyme-linked immunosorbent assay (ELISA) methods have been developed to confirm prior exposure to VHSV in the United States by detecting antibodies in fish serum (Millard and Faisal 2012; Millard et al. 2014; Wilson-Rothering et al. 2014, 2015). Enzyme-linked immunosorbent assay compares favorably with other antibody-detecting diagnostic tools. For example, the competitive ELISA that was developed by Millard et al. (2014), found 78.4% agreement with plaque neutralization testing. In 2014, Wilson-Rothering et al. published an antinucleocapsid-blocking ELISA that is able to detect nonneutralizing VHSV antibodies with greater sensitivity and specificity than a virus neutralization assay does. Although ELISA is gaining momentum as a useful diagnostic tool for VHSV, there are still knowledge gaps. Notably, the diagnostic performance characteristics of VHSV ELISA remain poorly understood for many fish species that are susceptible to VHSV, as do the kinetics of the antibody response that ELISA measures.

Although in principle the blocking ELISA can be applied to any species of fish, the performance characteristics of the assay in Northern Pike have not been assessed nor have cutoffs for diagnostic testing been determined. Therefore we examined antibody development over the course of disease in experimentally infected Northern Pike to examine the rate and timing of antibody development compared with the appearance of clinical signs, establish an inhibition cutoff threshold in Northern Pike, and thereby assess the applicability of the test to this economically important sport fish.

METHODS

Fish.— The fish were obtained at 6 months posthatch from the Wild Rose State Fish Hatchery in Wisconsin, USA. All of the fish were confirmed free of significant pathogens, including VHSV, by following the certified protocols in the American Fisheries Society Bluebook testing guidelines (Batts and Winton 2014).

The Northern Pike were allowed to acclimate for 6 months[1] in 200-L circular plastic tanks at a maximum density of 15 fish per tank in a recirculating system consisting of cycled deionized water with supplemental filtration and aeration. The deionized water was treated with a water conditioner (SeaChem Laboratories, Madison, Georgia) to remove residual hardness and to seed the biofilter with beneficial bacteria. The ammonia source for the fishless cycle was ammonium chloride (Millipore-Sigma, Saint Louis, Missouri). Frequent water testing was conducted to ensure a completed cycle prior to adding the fish to the tanks. After the fish were added to tanks, an automated light timer maintained a 12 h light : 12 h dark cycle. Daily water changes of at least 5% of the total tank volume were performed according to the recommendations by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin–Madison (IACUC approval V005768-A01). The fish were fed 2.0 mm pellets (Bio-Oregon, Westbrook, Maine) by an automatic feeder three times per day throughout the study. Water temperature was recorded daily and lowered from 17°C to 11 ± 1°C at a rate of 1°C per day before the start of the infection trials to mimic the temperature at which VHSV is most infective (Hershberger et al. 2013). Ammonia, nitrite, nitrate, and pH in each tank were tested and recorded weekly (the water quality parameters were maintained at approximately 0.25, 1.0, 20 mg/L, and 7.2, respectively).

Prior to VHSV exposure, the fish were anesthetized with a dose of 100 mg/L tricaine methanesulfonate

[1]The fish were held for this amount of time prior to experimental infection to ensure acclimatization and to obtain the necessary administrative clearances.

(MS-222; Syndel USA, Ferndale, Washington) that was buffered 1:1 (volume basis) with sodium bicarbonate (Millipore-Sigma, Saint Louis, Missouri) and marked with two visible implant elastomer tags (Northwest Marine Technology, Shaw Island, Washington) such that all of the individuals were uniquely identifiable. A baseline blood draw of 0.5 mL from the caudal tail vein of each fish was then performed. The blood samples were collected by using a 22G needle and syringe with the fish on a recirculating wet table, and the samples were then transferred to glass no-additive blood tubes (VWR International, Radnor, Pennsylvania) and inverted several times to induce clotting. The blood samples were allowed to clot overnight at 5°C then centrifuged for 15 min at $1,947 \times g$. The serum was separated and stored in cryovials at −80°C until testing by ELISA.

Culture and verification of the Great Lakes strain MI03 of VHSV.— Viral culture and quantification were performed at the La Crosse Fish Health Center in Onalaska, Wisconsin. Briefly, a Great Lakes strain VHSV-IVb isolate (confirmed by a reverse transcription PCR of a 946-base-pair diagnostic portion of the viral nucleoprotein gene and Sanger sequencing prior to the initiation of the study) was propagated by using a multiplicity of infection of less than 0.1 on endothelial progenitor cells that were grown in T75 tissue culture flasks with Minimum Essential Media-10 (Thermo Fisher Scientific, Waltham, Massachusetts) growth media. After a 100% cytopathic effect was observed, the virus stock was harvested from the flasks by scraping to dislodge the cells and media. These suspensions were pooled, centrifuged at $1,800 \times g$ for 10–15 min at 4°C to remove cellular debris, aliquoted, and frozen at −80°C. The virus was then quantified by serial dilution, and aliquots of 3.75 mL each suspended in Minimum Essential Media-10 growth medium were stored at −80°C for use in inoculation experiments. Using the tissue culture infectious dose that produced a 50% endpoint (TCID50; the dilution of virus-containing sample that infects 50% of tissue culture samples) the final concentration of the virus was 4.74×10^8 TCID50/mL (Binder 2017).

Experimental infection of Northern Pike.— Acclimatized Northern Pike (12 months old; average length 27.94 cm) were infected with 5×10^5 PFU/mL of VHSV by intraperitoneal injection (IP).[2] The fish were separated into three tanks of six to eight fish each, and six additional fish were kept in a separate tank as controls. The IP fish (23 fish in three tanks) were anesthetized with 100 mg/L MS-222 buffered 1:1 with sodium bicarbonate and then injected with a volume of 0.5 mL per fish. The control fish were mock-infected under the same conditions with cell culture media, Minimum Essential Media-10. After exposure, the fish were maintained at a water temperature of 11 ± 1°C. Daily monitoring during the experimental period included tank water temperature, observing fish for signs of disease, and recording mortalities. Euthanasia was warranted for fish that showed markedly abnormal swimming behavior, severe lethargy, severely decreased gill activity, severe anemia, and excessive bloating and/or hemorrhaging. The fish were euthanized with an overdose of MS-222 (200 mg/L) buffered 1:1 with sodium bicarbonate for 10 min.

Nonlethal blood samples were collected from the caudal vein of surviving fish, including controls, weekly for up to 12 weeks (84 d) postinfection. The blood collection and sample processing were performed as described above for the baseline blood draw. The fish that were euthanized prior to week 12 were only bled prior to euthanasia if the timing aligned with the weekly sampling schedule. On day 84, after the final weekly blood draw, all of the remaining fish were euthanized with an overdose of MS-222.

Antibody detection by competitive ELISA.— The blocking ELISA method that was developed by Wilson-Rothering et al. (2014) was used. Coating antigen was made from purified virus grown on endothelial progenitor cells, the same cell line that was used to culture the virus for experimental infection. Immulon II HB (Fisher Scientific, Hampton, New Hampshire) flat-bottomed 96-well plates were coated with 100 µL of antigen that was diluted at 1:200 in coating buffer in alternating positive and negative antigen rows. The coated plates were stored at −20°C until they were ready to use.

On the day of testing, the serum samples were first thawed at room temperature, then heated for 30 min at 45°C in a water bath to inactivate the complement, then centrifuged at $1,947 \times g$ for 15 m, and finally diluted at 1:8 in wash buffer to reduce nonspecific binding. Antigen and blocking buffer from a thawed ELISA plate were then removed, and 50 µL of diluted controls and Northern Pike serum was added to the positive and negative antigen wells. The plate was incubated for 30 min at 37°C, after which 50 µL of monoclonal antibody (Aquatic Diagnostics, Sterling, Scotland; conjugated by American Qualex, San Clemente, California) diluted 1:6,000 in blocking buffer (phosphate-buffered saline) was added to all of the wells containing sera. The plate was incubated for 90 min at 37°C and then washed three times. One hundred microliters of Sure-Blue tetramethylbenzidine substrate (KPL, Gaithersburg, Maryland) was then added to each well, and the plate developed for approximately 15 min at 37°C. One hundred microliters of tetramethylbenzidine Stop Solution (KPL, Gaithersburg, Maryland) was added to terminate the reaction. The ELISA plate was then read

[2]Initially, an additional group of eight Northern Pike was infected by static immersion (SI; 90 min in 30 L of aerated aquarium with 45.86 mL of virus stock, 5×10^5 PFU/mL of VHSV). [Correction added on March 3, 2020, after first online publication: This footnote has been corrected.] However, the preliminary data indicated that this group was not successfully infected. Therefore, the SI group was not included in final analyses.

by using an absorbance microplate reader at an optical density (OD) of 450 nm. The OD readings were adjusted to eliminate background by subtracting the readings from the negative antigen wells from those from the positive antigen wells. The ELISA results were reported as percentage of inhibition (%I) and normalized to correct for any overdevelopment of negative samples by multiplying by the value of the negative control OD divided by the highest sample OD on each plate (Wright et al. 1993). The negative control consisted of pooled serum from confirmed-negative hatchery-reared Brown Trout from the Wild Rose State Fish Hatchery in Wisconsin.

Statistical analyses.— The data analyses were performed using R version 3.3.3 (R Core Team 2017). To determine the optimal positive and negative percent inhibition threshold, a receiver-operating characteristic (ROC) curve was constructed. All of the ELISA results (%I) from every fish at each point (including those from the baseline samples) combined with binary viral exposure status of the fish (negative = not exposed to VHSV, positive = exposed to VHSV) were used to form the ROC curve. A threshold %I value was chosen as the cutoff that maximized both sensitivity (true positive results) and specificity (true negative results). Kaplan–Meier survival curves were used to display the survival probability of IP and control fish at postinfection. The groups were compared by using a log-rank test. A *P*-value of less than 0.05 was considered statistically significant. To account for potential confounders (exposure status, survival time, and time to development of clinical signs of disease), a Cox proportional hazard model was also constructed. The Kruskal–Wallis rank sum test was used to analyze the difference in %I between infected and noninfected fish.

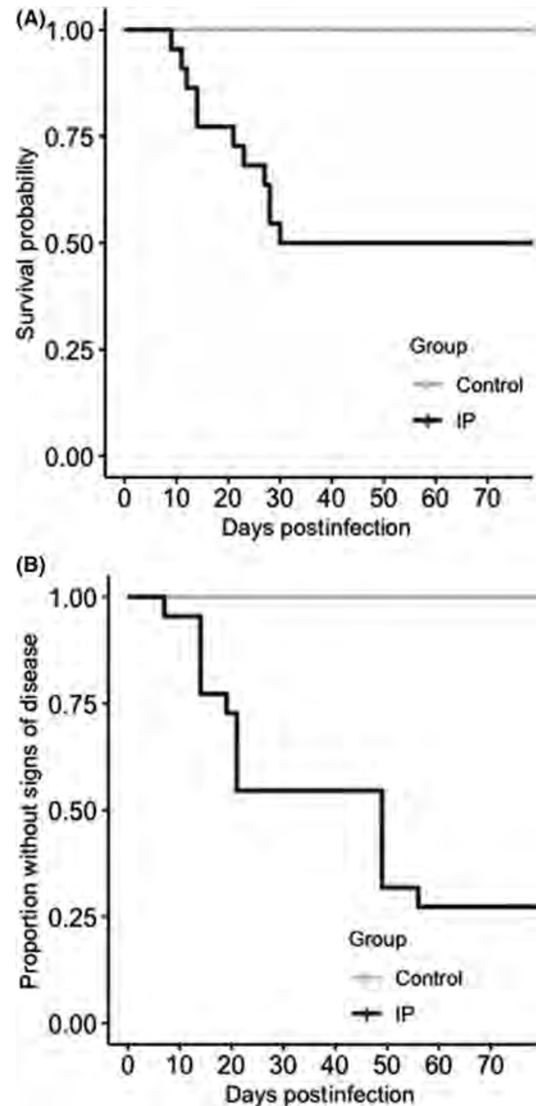

FIGURE 1. **(A)** The survival probabilities for both experimental groups of Northern Pike (control and IP) and **(B)** the Kaplan–Meier curve showing the proportion of Northern Pike without clinical signs of disease over time. The log-rank tests showed a significant difference between the two groups ($\chi^2 = 16.1$, df = 1, $P = 0.00005$). About 50% of IP-infected fish showed clinical signs of disease by 21 d postinfection.

RESULTS

Survival and Development of Clinical Signs of Disease

The survival probabilities for both experimental groups (control and IP) are shown in Figure 1A. The log-rank tests showed a significant difference between the two groups ($\chi^2 = 15.2$, df = 1, $P = 0.00009$). The control group had the highest percentage of survival to the end of the experiment (100%), followed by the IP group with 50% survival. The median survival time for the IP group was 69 d postinfection. The Cox proportional hazard analysis showed a significant difference in risk of death between the control and IP groups ($P = 4.03 \times 10^{-7}$).

Figure 1B shows the probability that a Northern Pike remained nonclinically affected (i.e., did not display clinical signs of disease) for each group over time. No signs of disease were observed in the control group at any point during the postinfection observation period. However, the exposed group showed clinical signs including erythema, exophthalmia, anemia, bloated abdomen, and abnormal swimming behavior. The log-rank tests showed a significant difference between the two groups ($\chi^2 = 16.1$, df = 1, $P = 0.00005$). The infected group showed an increase in the probability of development of clinical signs of disease over time, with about 50% of the Northern Pike displaying signs of disease by 21 d postinfection. The Cox proportional hazard analysis showed a slight decrease in risk of death when signs of disease were observed (Hazard ratio 0.98, 95% confidence interval, 0.97–1.00, $P = 0.02$), i.e., the fish that showed clinical signs of disease survived

longer than those that did not show clinical signs of disease did. As shown in Figure 1, almost 25% of the infected fish had already died during the acute stage of infection (prior to the peak development of clinical signs at 21 d postinfection), likely explaining this observation. The other potential confounders that were examined were not significant.

Kinetics of the Antibody Response

Figure 2 shows the average percentage of inhibition by ELISA for both the IP and control groups over the 12 weeks postinfection, including the baseline samples that were taken prior to infection. The %I for the control fish ranged from 0% to 59.9% (average, 36.0%). At baseline, the fish that were infected by IP injection had %I values that ranged from 5.3% to 66.1% (average, 42.7%), and %I ranged from 0% to 89.0% (average, 51.0%) during the postinfection period. The standard errors for the control and IP groups were 1.53% and 1.44%, respectively. Furthermore, the average rate of change per week for each group was −0.15% and 0.42%, respectively, over the course of 12 weeks.

ELISA Diagnostic Performance Characteristics

Table 1 shows a summary of the VHSV ELISA results, with Northern Pike divided into either infected (IP) or uninfected (control) groups. To avoid false negatives, virus isolation was not performed as a confirmatory test, as viral titers varied at time of death and most likely would be low or even absent (based on the preliminary trials that preceded this study). Given the 41.3% threshold, 112 of 139 serum samples (80.5%) from the IP injection group were positive, as determined by ELISA. The Kruskal–Wallis rank sum test showed a significant difference in the %Is between the infected and the uninfected groups ($\chi^2 = 43.6$, df = 1, $P = 3.9 \times 10^{-11}$). Figure 3A shows the results of the ROC analysis of ELISA sensitivity and specificity. The area under the ROC curve was 0.7613. A %I value of ≥41.3% to demarcate positive samples and <41.3% to demarcate negative samples maximized the accuracy of the assay. With these cutoff values, the ELISA performed at a sensitivity of 80.5% (95% confidence interval, 73–87%) and a specificity of 63.2% (95% confidence interval, 52–73%). The positive predictive value of the ELISA for infected fish was 78% (95% confidence interval, 70–84%) and the negative predictive value was 67% (95% confidence interval, 56–77%). The positive likelihood ratio was 2.19 (95% confidence interval, 1.64–2.92) and the negative likelihood ratio was 0.31 (95% confidence interval, 0.21–0.45).

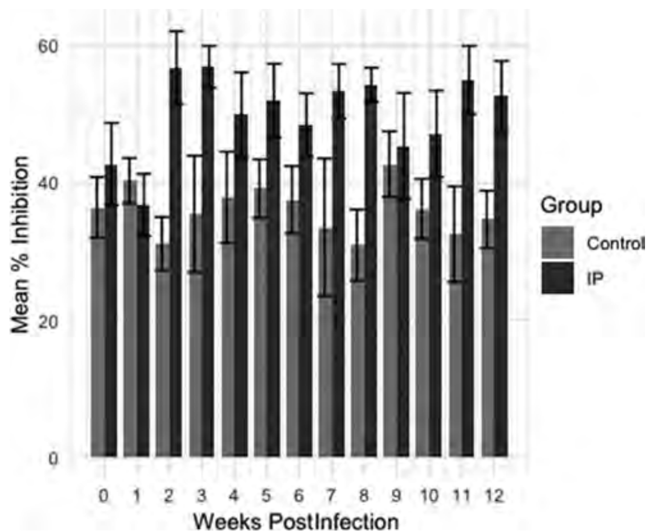

FIGURE 2. Comparison of average percent inhibitions for each experimental group over the course of infection. The error bars represent standard error of the mean.

Figure 3B shows the results of an additional ROC analysis of ELISA sensitivity and specificity that was completed by using a subset of data from only weeks 2–12 during the postinfection period. Using the same threshold as is described above, the area under the ROC curve was 0.7860, with a sensitivity of 83% (95% confidence interval, 74–90%) and specificity of 62% (95% confidence interval, 47–76%).

ELISA Kinetics

Positive ELISA results were detectable during all 12 weeks postinfection for the experimentally infected group. The highest percentage of positive results occurred during weeks 5 and 8 of the postinfection period (Figure 4). A comparison of standard error of the mean over time shows that the average %I of the infected group differed significantly from that of the control group during weeks 2–3, 5–8, and 10–12 postinfection (Figure 2).

DISCUSSION

Our results show that Northern Pike that were infected with VHSV by intraperitoneal injection showed a 50% survival rate and the development of a sustained antibody response over the course of a 12-week postinfection period. The survival rates for the infected fish were significantly lower than those of the mock-infected controls

TABLE 1. The 2 × 2 table of VHSV ELISA results for fish of known infection status.

	True positive	True negative	Total
ELISA positive	105	26	131
ELISA negative	115	65	180
Total	220	91	311

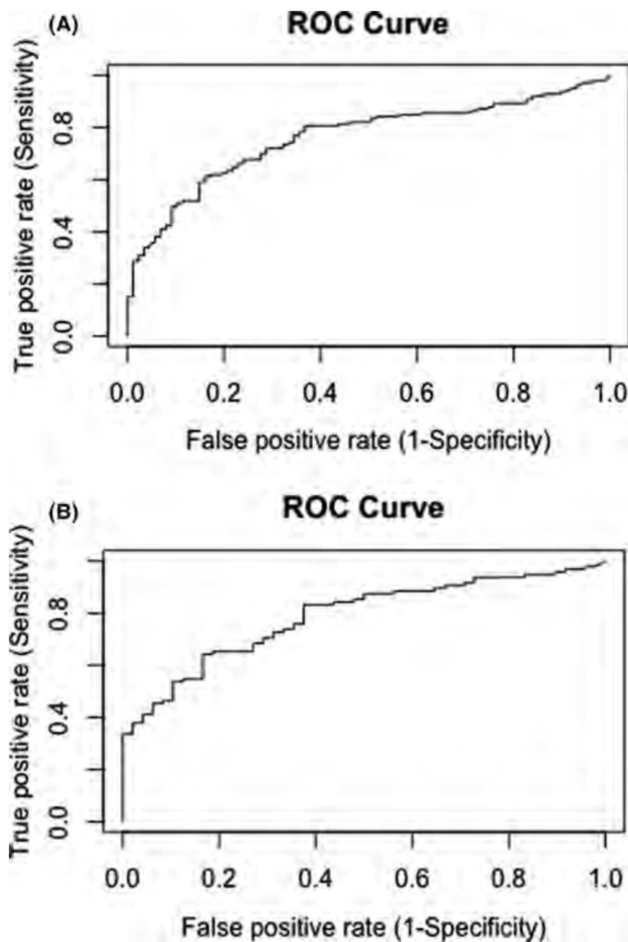

FIGURE 3. **(A)** Receiver operating characteristic (ROC) curve showing the true positive rate for Northern Pike serum that was tested at a 1:8 dilution by VHSV ELISA. The data that were used to form this curve consisted of 139 infected fish and 87 uninfected fish. The area under the curve is 0.7613. At the optimum threshold of 41.3% inhibition, the sensitivity of the ELISA is 80.5% and specificity is 63.2%. If the threshold is altered to maximize specificity, the new threshold is 58.2% inhibition, with a sensitivity of 34.5% and specificity of 95.4%. Also shown is **(B)** an alternative ROC curve showing the true positive rate for the Northern Pike serum that was tested at a 1:8 dilution by VHSV ELISA. A subset of the data from weeks 2–12 postinfection for both controls and IP-infected fish were used to form this curve. The data consisted of 95 infected fish and 48 uninfected fish. The area under the curve is 0.7862.

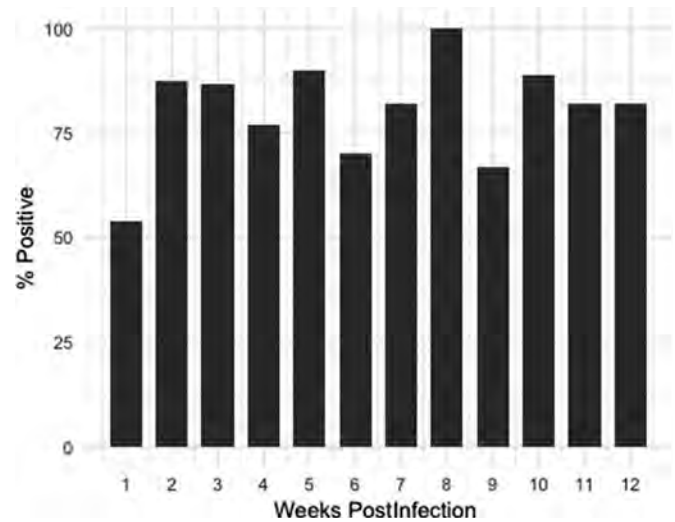

FIGURE 4. Percentage of infected Northern Pike that tested positive by ELISA during each week postinfection. The highest percentage of positive cases occurred during week 5 and week 8 (90% and 100%, respectively) .

(100%). The onset of clinical signs in the majority of infected Northern Pike occurred at 3 weeks postinfection. The globally optimal sensitivity (80.5%) and specificity (63.2%) of the VHSV ELISA was achieved by setting the inhibition cutoff at 41.3%. However, alternative cutoffs can achieve substantially higher sensitivity or specificity, which may be advantageous for certain applications.

It is noteworthy that we successfully replicated the blocking ELISA method by Wilson-Rothering et al. (2014) with a few minor alterations and used it to detect the presence of nonneutralizing anti-VHSV antibodies in experimentally infected Northern Pike, which were not a species that was used to develop or assess the method. Notable alterations in our protocol included increasing the serum sample test dilution and the centrifugation of the serum prior to testing. Both of these changes helped to reduce background (nonspecific binding) in the negative antigen wells. Nonspecific binding seemed to occur more frequently in the serum from Northern Pike than has been observed in other species that have been tested previously. Only 6 of 78 control samples (7.6%) had background ODs > 0.1 in the negative antigen well, whereas 33 of 148 fish (22.2%) that were infected by IP injection had background ODs > 0.1 at the 1:8 dilution. The high level of nonspecific binding that was observed in this study could have been caused by the inoculation procedure. In a study conducted by Güven et al. (2014), for example, nonspecific binding in a human autoantibody ELISA correlated with inflammatory markers in serum.

An inhibition threshold of 41.3%, which maximized overall test accuracy, yielded a sensitivity of 80.5% and a specificity of 63.2%. These values are lower than the values of 96.4% and 88.2%, respectively, that were reported by Wilson-Rothering et al. (2014). These differences could indicate factors that are unique to different esocid species, in that Northern Pike were not included in the study by Wilson-Rothering et al. (2014). Similarly, Millard et al. (2014) found that competitive ELISA and a plaque neutralization test had strong agreement but a lack of a gold standard in that study precluded a formal assessment of sensitivity and specificity. Therefore, it is noteworthy that

the area under the ROC curve improved marginally when the fish from weeks 2–12 postinfection were analyzed separately (Figure 3; The area under the curve was 0.7613 and 0.7862, respectively), indicating that ELISA sensitivity and specificity improves when fish are tested during the optimum window of antibody detection. Using the 41.3% cutoff for Northern Pike reported herein would favor sensitivity (i.e., detecting true positives) over specificity (i.e., detecting true negatives), although neither value is ideal. However, depending on the purpose for which the test is used, alternative cutoffs could be chosen. For example, the cutoff could be lowered to increase sensitivity in a situation where it is important to maximize detection of positive fish (e.g., prior to translocating fish into a VHSV-free water body), albeit at the expense of elevating the false positive rate. Otherwise, the cutoff could be raised to increase specificity in a situation where false positive results would be costly (e.g., prior to destroying fish or eggs). The choice of a cutoff should, in other words, be dictated by the purpose of testing.

Fish that were infected by the IP route developed detectable antibodies by 1 week postinfection with the most consistency in detectable positive results from 2 to 12 weeks postinfection. Notably, we documented high interindividual variation in ELISA positivity both among fish and within fish over time. Therefore, the ELISA assay in this study is more suited to assessing the population status of VHSV exposure rather than assessing the infection history of an individual fish. Indeed, its best use for management may be to compare the sero-status of species and populations over space and time.

In this light, our results also show that clinical signs and ELISA positivity rates peak at approximately 2–8 weeks postviral exposure. This timing corresponds to the dynamics of the disease in natural populations, which is surely more complex, and provides some calibration for the interpretation of positive results. For example, the ELISA assay described herein would probably be less useful for assessing exposure of Northern Pike to VHSV immediately after the introduction of the disease but more useful several weeks or months afterwards. Given the time limits of our study, the duration for which Northern Pike remain ELISA-positive following VHSV exposure remains unclear, as do the physiological and environmental factors that might affect that duration. Nevertheless, even an approximate knowledge of the timing of VHSV exposure in natural populations of Northern Pike (or other species) could improve management decisions, especially given the potentially rapid turnaround time of the assay.

Conclusion

A competitive ELISA method that was developed by Wilson-Rothering et al. (2014) for detecting antibodies to VHSV is repeatable and performs with moderate sensitivity and specificity in Northern Pike (80.5% and 63.2%, respectively) when a 41.3% inhibition threshold is chosen, but either value can be improved by lowering or raising this threshold, respectively, as warranted by the purpose for which the test is used. In experimentally infected Northern Pike, nonneutralizing anti-VHSV antibodies developed by 1 week postinfection and were detectable through all 12 weeks postinfection, but the highest likelihood of detection occurred from weeks 2 to 8 postinfection, which aligned with the development of clinical signs. The potential uses of this assay in Northern Pike include, but are not limited to, testing wild Northern Pike for general VHSV surveillance, testing wild Northern Pike in hatchery source waters, testing Northern Pike that are used as broodstock to supply hatcheries, or testing of Northern Pike prior to translocation. The study also outlines methods that can be used to identify optimal thresholds and sample dilutions for other situations and species.

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