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Article

Assessment of a serologic diagnostic test and kinetics of antibody development in Northern Pike *Esox lucius* 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 non-lethal serological methods that can have significantly shorter turn-around 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 non-neutralizing anti-nucleocapsid antibodies to VHSV in Northern Pike *Esox lucius*. Juvenile Northern Pike were experimentally infected with VHSV by intraperitoneal injection. Infected fish were monitored for 12 weeks for signs of disease, and weekly serum samples were obtained. Analysis of survival data showed that mortality occurred significantly more quickly in inoculated fish than in control fish. Fish infected by injection showed a significant increase in antibody response by two weeks post infection. However, variation in the rate and pattern of antibody response among infected fish was high at any given time point. The optimum window of detection of antibodies in Northern Pike is 2 to 12 weeks post-infection, which generally follows the median time to appearance of clinical signs (21 days post-infection). Receiver-operator 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 use of the test for specific management goals. Results of this study offer insights into the disease progression and

immune kinetics of VHSV, including inter-individual variation, which will aid in management of this economically important virus.

Introduction

Viral hemorrhagic septicemia virus (VHSV) is a rhabdovirus (*Rhabdoviridae*: *Novirhabdovirus*) affecting a diversity of fish species worldwide (Kim and Faisal 2011; Millard and Faisal 2012; Millard et al. 2014; Wilson-Rothering et al. 2014, 2015; Wolf 1988). 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 (Faisal et al. 2012; Kim and Faisal 2010a, 2010b; Olsen 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 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 EPC) for 14 days (although a positive result may appear sooner), followed by a 14 day 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 turn-around-time (TAT) for results, and detects current infection but not prior exposure. Rapid antibody detection methods, such as serologic methods, are non-lethal and could reduce TAT 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 et al. 2014; Millard and Faisal 2012; Wilson-Rothering et al. 2014, 2015). ELISA compares favorably to other antibody-detecting diagnostic tools. For example, the competitive ELISA developed by Millard et al. 2014, found 78.4% agreement with plaque neutralization testing. In 2014, Wilson-Rothering et al. published an anti-nucleocapsid-blocking

ELISA able to detect non-neutralizing VHSV antibodies with greater sensitivity and specificity than a virus neutralization assay. Although ELISA is gaining momentum as a useful diagnostic tool for VHSV, knowledge gaps still exist. 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 this blocking ELISA can in principle 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. We therefore examined antibody development over the course of disease in experimentally-infected Northern Pike to examine the rate and timing of antibody development compared to 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.

<A>Methods

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

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. Deionized water was treated with a water conditioner (SeaChem Laboratories, Madison, Georgia) to remove residual hardness and to seed the bio-filter 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 fish to tanks. After fish were added to tanks, an automated light timer maintained a 12-hour light cycle. Daily water changes of at least 5% of total tank volume were performed following recommendations by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Madison (IACUC approval number V005768-A01). Fish were fed 2.0 mm pellets (Bio-Oregon, Westbrook, Maine) by

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

automatic feeder 3 times per day throughout the study. Water temperature was recorded daily and lowered from 17°C to 11±1°C at a rate of 1ºC per day before the start of infection trials to mimic the temperature at which VHSV is most infective (Hershberger et al. 2013). Ammonia, nitrite, nitrate, and pH of each tank were tested and recorded weekly (water quality parameters were maintained at approximately 0.25ppm, 1ppm, 20ppm, and 7.2, respectively).

Prior to VHSV exposure, fish were anesthetized with a dose of 100mg/L tricaine methanesulfonate (MS-222; Syndel USA, Ferndale, Washington) buffered 1:1 with sodium bicarbonate (Millipore-Sigma, Saint Louis, Missouri), marked with two visible implant elastomer tags (Northwest Marine Technology, Inc., Shaw Island, Washington) such that all individuals were uniquely identifiable, and a baseline blood draw of 0.5mL from the caudal tail vein was performed. Blood samples were collected using a 22G needle and syringe with the fish on a recirculating wet table, then samples were transferred to glass no-additive blood tubes (VWR International, Radnor, Pennsylvania) and inverted several times to induce clotting. Blood samples were allowed to clot overnight at 5°C then centrifuged for 15 min at 1947 x *g*. Serum was separated and stored in cryovials at -80°C until testing by ELISA.

Culture and verification of 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 reverse transcription PCR of a 946 base pair diagnostic portion of the viral nucleoprotein gene and Sanger sequencing prior to initiation of the study) was propagated using a multiplicity of infection of less than 0.1 on EPC cells grown in T75 tissue culture flasks with Minimum Essential Media-10 (MEM; Thermo Fisher Scientific, Waltham, Massachusetts) growth media. After 100% cytopathic effect was observed, virus stock was harvested from flasks by scraping to dislodge cells and media. These suspensions were pooled, centrifuged at 1800 x g for 10-15 minutes at 4°C to remove cellular debris, aliquoted, and frozen at -80°C. Virus was then quantified by serial dilution (final concentration of cultured virus: 4.74×10^8 $TCID_{50}$/mL; Binder 2017) and aliquots of 3.75mL each suspended in MEM-10 growth medium were stored at -80° C for use in inoculation experiments.

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]. Northern Pike were separated into 3 tanks of 6 to 8 fish each and six additional fish were kept in a separate tank as controls. IP fish (23 fish in 3 tanks) were anesthetized with 100mg/L MS-222 buffered 1:1 with sodium bicarbonate and then injected with a volume of 0.5 mL per fish. Control fish were mock infected under the same conditions with cell culture media, MEM. Post-exposure, 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 showing markedly abnormal swimming behavior, severe lethargy, severely decreased gill activity, severe anemia, and excessive bloating and/or hemorrhaging. Fish were euthanized with an overdose of MS-222 (200mg/L) buffered 1:1 with sodium bicarbonate for 10 minutes.

Non-lethal blood samples were collected from the caudal vein from surviving fish, including controls, weekly for up to 12 weeks (84 days) post infection. Blood collection and sample processing were performed as described above for the baseline blood draw. Fish 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 remaining fish were euthanized with an overdose of MS-222.

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

On the day of testing, serum samples were first thawed at room temperature, then heated for 30 minutes at 45°C in a water bath to inactivate complement, then centrifuged at 3700 rpm for 15 minutes, 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 pike serum was added to positive and negative antigen wells. The plate was incubated for 30 minutes at 37°C, after which 50 µL of monoclonal antibody (Aquatic Diagnostics, Sterling, Scotland; conjugated by American Qualex, San Clemente, California)

[2] Initially, an additional group of 8 Northern Pike was infected by static immersion (SI; 90 minutes in 30 L of aerated aquarium with 45.86 mL of virus stock, $5x10^5$ PFU/mL of VHSV). However, preliminary data indicated that this group was not successfully infected. Therefore, the SI group was not included in final analyses.

diluted 1:6000 in blocking buffer (Phosphate Buffered Saline) was added to all wells containing sera. The plate was incubated for 90 minutes at 37°C, then washed three times. One hundred microliters of Sure-Blue tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, Maryland) was then added to each well and the plate developed for approximately 15 minutes at 37°C. One hundred microliters of TMB Stop Solution (KPL, Gaithersburg, Maryland) was added to terminate the reaction. The ELISA plate was then read using an absorbance microplate reader at an optical density (OD) of 450 nm. OD readings were adjusted to eliminate background by subtracting OD readings from negative antigen wells from positive antigen wells. ELISA results were reported as percent 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.— . Data analyses were performed using R version 3.3.3 (R Core Team 2017). To determine optimal positive and negative percent inhibition threshold, a receiver-operator characteristic (ROC) curve was constructed. All ELISA results (%I) from every fish at each time point (including 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 survival probability of IP and control pike post-infection. The groups were compared 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 percent inhibition of infected fish compared to not infected fish.

<A>Results

<B>Survival and development of clinical signs of disease

Survival probabilities for both experimental groups (control and IP) are shown in Figure 1a. Log-rank tests showed a significant difference between the two groups (X^2= 15.2, df=1, *P* =

0.00009). The control group had the highest percent survival to the end of the experiment (100%) followed by the IP group with 50% survival. Median survival time for the IP group was 69 days post-infection. Cox proportional hazard analysis showed a significant difference in risk of death between control and IP groups (P=4.03x10^{-7}).

Figure 1b shows the probability that a fish remained non-clinically 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 post-infection. However, the exposed group showed clinical signs including erythema, exopthalmia, anemia, bloated abdomen, and abnormal swimming behavior. Log-rank tests showed a significant difference between the two groups (X^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 pike displaying signs of disease by 21 days post-infection. 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, P=0.02), i.e. pike showing clinical signs of disease survived longer than those that did not show clinical signs of disease. As shown in Figure 1, almost 25% of infected fish had already died during the acute stage of infection (prior to the peak development of clinical signs at 21 days post-infection), likely explaining this observation. Other potential confounders examined were not significant.

<B>Kinetics of the antibody response

Figure 2 shows average percent inhibition by ELISA for both IP and control groups over the 12 weeks post-infection, including baseline samples taken prior to infection. Control fish percent inhibitions ranged from 0% to 59.9% (average, 36.0%). At baseline, fish infected by IP injection had percent inhibitions of 5.3% to 66.1% (average, 42.7%), and a range of 0% to 89.0% (average, 51.0%) inhibition post-infection. 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.

<B>ELISA diagnostic performance characteristics

Table 1 shows a summary of 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 preliminary trials that precede this study). Given the 41.3% threshold, 112 of 139 serum samples (80.5%) from the IP injection group were positive by

ELISA. Kruskal Wallis rank sum tests showed a significant difference in the percent inhibitions of infected compared to uninfected groups (χ^2=43.6, df=1, P = 3.9x10^{-11}). Figure 3a shows the results of ROC analysis of ELISA sensitivity and specificity. The area under the ROC curve was 0.7613. A percent inhibition value of ≥41.3% to demarcate positive samples and <41.3% to demarcate negative samples maximized accuracy of the assay. With these cutoff values, the ELISA performed at a sensitivity of 80.5% (95% confidence interval, 73% to 87%) and a specificity of 63.2% (95% confidence interval, 52% to 73%). The positive predictive value of the ELISA for infected fish was 78% (95% confidence interval, 70% to 84%) and the negative predictive value was 67% (95% confidence interval, 56% to 77%). The positive likelihood ratio was 2.19 (95% confidence interval 1.64 to 2.92) and the negative likelihood ratio was 0.31 (95% confidence interval 0.21 to 0.45).

Figure 3b shows the results of an additional ROC analysis of ELISA sensitivity and specificity using a subset of data from weeks 2-12 post infection only. The area under the ROC curve was 0.7860 with a sensitivity of 83% (95% confidence interval 74% to 90%) and specificity of 62% (95% confidence interval 47% to 76%) using the same threshold as described above.

<*B*>ELISA kinetics

Positive ELISA results were detectable during all 12 weeks post infection for the experimentally-infected group. The highest percentage of positive results occurred during weeks 5 and 8 post-infection (Figure 4). Comparison of standard error of the mean over time shows that the average percent inhibition of the infected group differed significantly from the control group during weeks 2-3, 5-8, and 10-12 post-infection (Figure 2).

<A>Discussion

Our results show that Northern Pike infected with VHSV by intraperitoneal injection show 50% survival and development of a sustained antibody response over the course of 12 weeks post infection. Survival rates for infected fish were significantly lower than mock-infected controls (100%). Onset of clinical signs in the majority of infected Northern Pike occurred at 3 weeks post-infection. 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 presence of non-neutralizing anti-VHSV antibodies in experimentally-infected Northern Pike, which were not a species used to develop or assess the method. Notable alterations in our protocol included increasing the serum sample test dilution and centrifugation of serum prior to testing. Both of these changes helped reduce background (non-specific binding) in negative antigen wells. Non-specific binding seemed to occur more frequently in Northern Pike serum than in other species previously tested. Only 6 of 78 control samples (7.6%) had background (optical densities [OD] >0.1 in the negative antigen well), whereas 33 of 148 fish (22.2%) infected by IP injection had background ODs at the 1:8 dilution. The high level of non-specific binding observed in this study could have been caused by the inoculation procedure. In a study conducted by Güven et al 2014, for example, non-specific binding in a human auto-antibody 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, reported by Wilson-Rothering et al (2014). These differences could indicate factors unique to different esocid species, in that Northern Pike were not included in the study by Wilson-Rothering et al. Similarly, Millard et al (2014) found that competitive ELISA and a plaque neutralization test had strong agreement, but lack of a gold standard in that study precluded formal assessment of sensitivity and specificity. It is therefore noteworthy that the area under the ROC curve improved marginally when fish from weeks 2-12 post infection were analyzed separately (Figure 3; AUC is 0.7613 and 0.7862, respectively), indicating 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. Or, the cutoff could be

raised to increase specificity in a situation where a 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 infected by the IP route developed detectable antibodies by 1 week post infection with the most consistency in detectable positive results from 2-12 weeks post infection. Notably, we documented high interindividual variation in ELISA positivity, both among fish and within fish over time. The ELISA assay in this study is therefore 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 approximately 2-8 weeks post-viral exposure. This timing corresponds to the dynamics of the disease in natural populations, which is surely more complex, and provides some calibration for interpretation of positive results. For example, the ELISA assay described herein would probably be less useful for assessing exposure of pike to VHSV immediately after VHSV introduction 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 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 TAT of the assay.

<B>Conclusion

A competitive ELISA method 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, non-neutralizing anti-VHSV antibodies developed by 1 week post infection and were detectable through all 12 weeks post infection, but the highest likelihood of detection occurred from weeks 2 to 8 post infection, which aligned with the development of clinical signs. Potential uses of this assay in

Northern Pike include, but are not limited to, testing of wild Northern Pike for general VHSV surveillance, testing of wild Northern Pike in hatchery source waters, testing of Northern Pike 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 other species.

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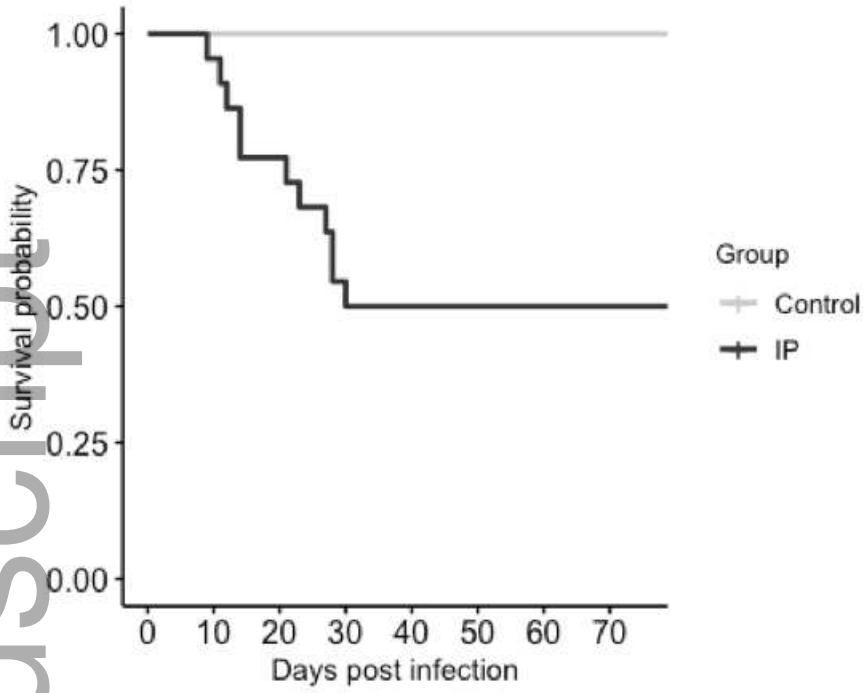
Table 1. 2X2 table of VHSV ELISA results for fish of known infection status.

	True Positive	True Negative	Total
ELISA Positive	105	26	131
ELISA Negative	115	65	180
Total	220	91	311



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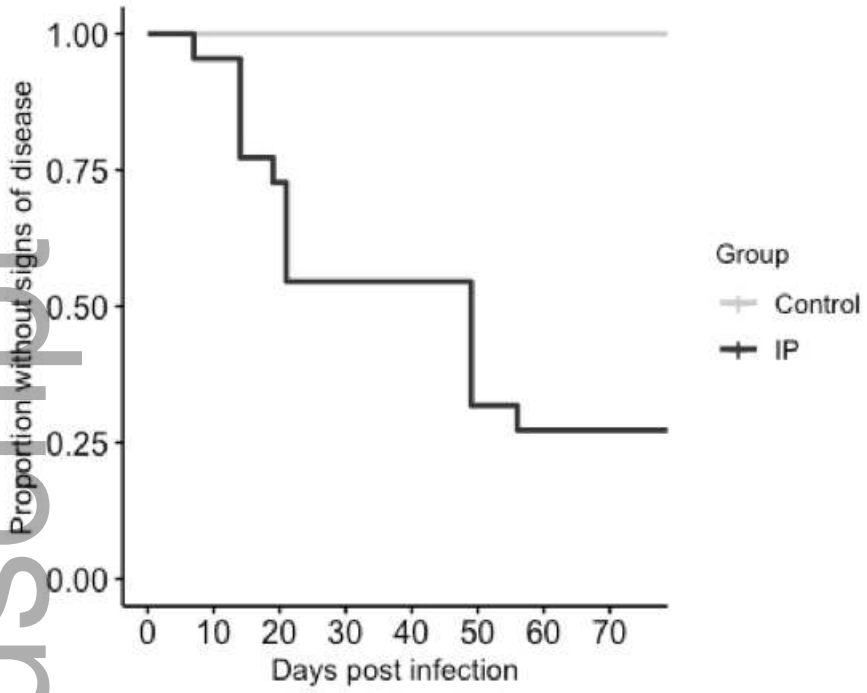

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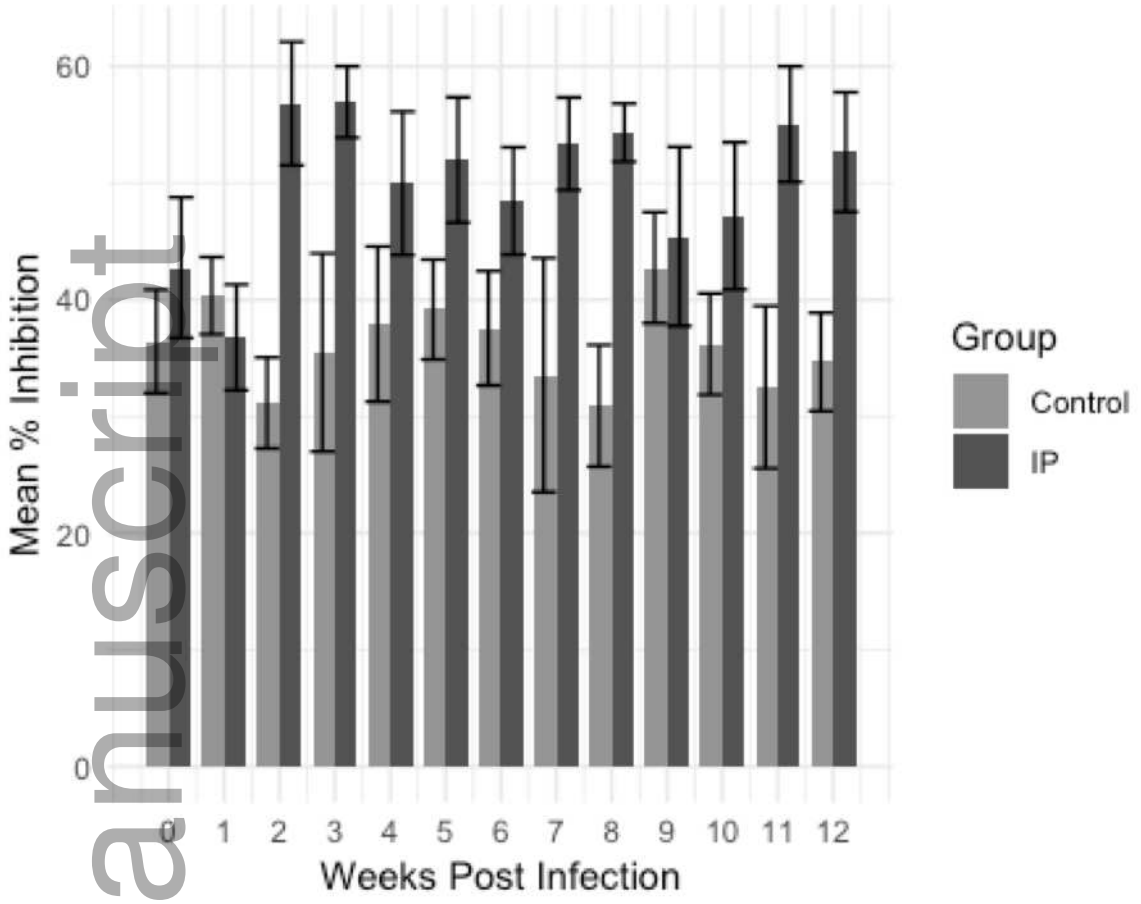

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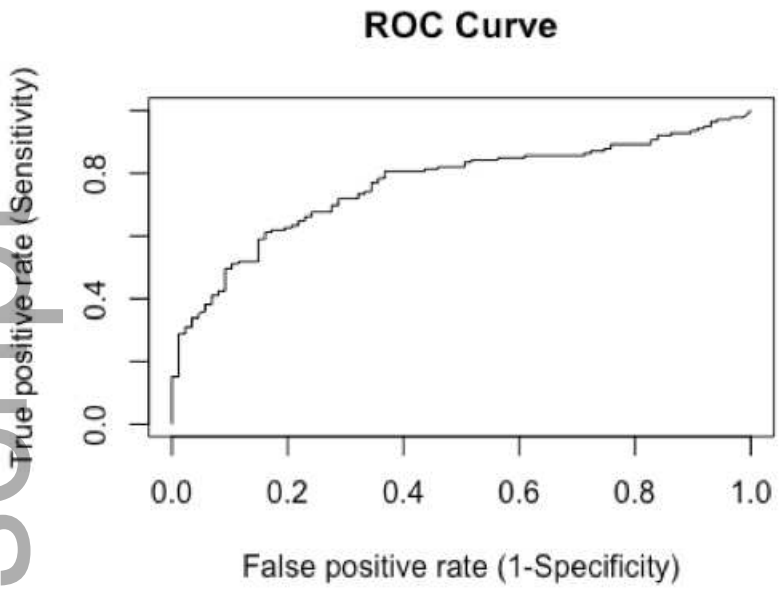

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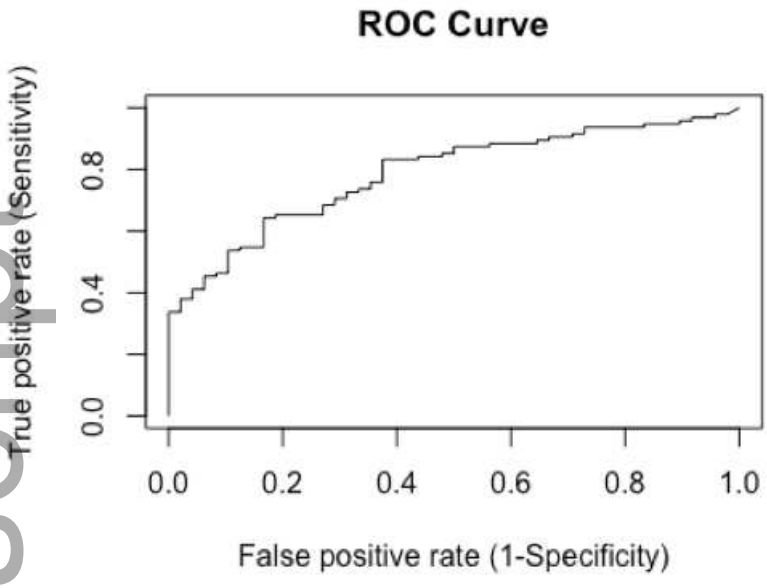

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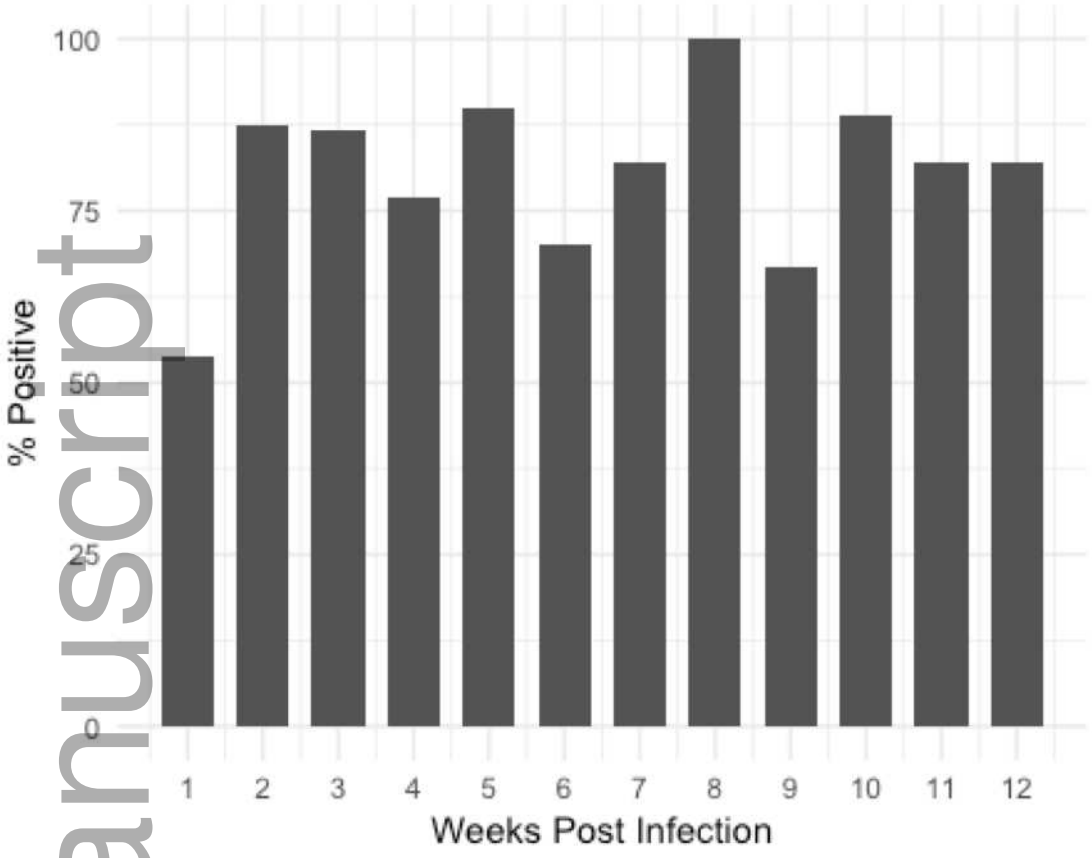

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