Great Lakes Fish and Wildlife Restoration Act
FINAL Project Report

Project Title: Development of Non-Lethal Sampling Method for Disease Detection

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Study Objectives:
The ultimate objective of this study is to determine the usefulness of nonlethal sampling for the diagnosis of fish diseases within a population. In specific, this study was performed with the following objectives:

Objective 1: To compare between lethal (kidneys and spleen) and non-lethal (blood, mucus, and uro-fecal swab) samples for the detection of viral hemorrhagic septicemia virus (VHSV) following a standardized experimental infection.

Objective 2: To compare between lethal (kidneys and spleen) and non-lethal (blood, mucus, and uro-fecal swab) samples for the detection of *Renibacterium salmoninarum* (*Rs*), the causative agent of bacterial kidney disease (BKD) following a standardized experimental infection.

Objective 3: To compare lethal and non-lethal sampling methods for detecting pathogens in naturally infected fish stocks and to determine if the pathogen/viral load plays a role in non-lethal detection efficacy.

Objective 4: To determine the best statistical approach for relating non-lethal diagnostic tests to prevalence of disease and to determine the appropriate number of fish to sample using non-lethal (imperfect) diagnostic tests to detect the prevalence of a disease in a subpopulation or population of fish.

Description of Tasks:

Objective 1: To compare between lethal (kidneys and spleen) and non-lethal (blood, mucus, and uro-fecal swab) samples for the detection of VHSV following a standardized experimental infection.

This experiment was performed on muskellunge (*Esox masquinongy*) with the following tasks:

Task 1.1 Waterborne challenge of muskellunge.

- VHSV-free certified muskellunge (6 months post-hatch) were infected by waterborne challenge in triplicate groups of 30 fish. Infectious tissue culture media of the Great Lakes VHSV-IVb strain MI03 was added to static tank water to create three different concentrations: 10^5 pfu ml^{-1} (high dose), 10^4 pfu ml^{-1} (intermediate dose) and 10^3 pfu ml^{-1} (low dose) to induce acute, subacute and chronic courses of the disease, respectively. Fish were held in water at 11 °C with the ascribed doses for 1.5 hours. A control tank of fish was mock infected with sterile tissue culture media and served as the negative control group.

Task 1.2 Sampling regime and monitoring of fish. Sampling by lethal and non-lethal methods was conducted in the following manner. Dead and moribund fish were collected daily every 8 hours. Sampling continued until all fish were tested.

- In the high VHSV dose group, 20 live fish were collected from each tank on days 1, 2, 3, 4, 5, 6, and 7. Blood, mucus, and uro-fecal swabs were initially collected from each fish, followed by collection of kidneys and spleen for lethal sample testing.
- In the intermediate VHSV dose group, 20 live fish were collected on days 1, 5, 7, 10, 14, and 21 days and samples collected as described for the high dose group.
- In the low VHSV dose group, 7 live fish were collected from each tank on day 1, followed by every 7 days for 70 days. Samples were collected as described for the high dose group.

Task 1.3 Non-lethal tissue sampling and detection of VHSV. Non-lethal tissue sampling was conducted by first anesthetizing fish at 100-150 ppm of tricaine methanesulfonate (MS-222).

- Peripheral blood was collected by venipuncture in the caudal vein, serum separated, and stored at -80 °C until assayed.
- Mucus was collected just caudal to the vent and along the first ray of the anal fin with a sterile scalpel blade, weighed, and stored at -80° C until assayed.
- Urine-Feces samples were obtained by gently pressuring the ventral abdominal wall anterior to the vent against the tip of sterile swab. The withdrawn urine-feces mixture was then suspended in 500 µl of saline in microcentrifuge tubes and stored at -20° C until assayed.

Task 1.4 Lethal sampling.

- Fish were euthanized by an overdose of MS-222. Kidney and spleen samples were collected and placed in individual Whirl-Paks.

Task 1.5 Diagnostic assays for VHSV detection.

- A battery of VHSV diagnostic assays were performed on collected samples. These assays include virus re-isolation on Epitheliosum papulosum cyprini (EPC) cell line, nested RT-PCR, and real time RT-PCR.
- In addition, serological assays optimized for the detection of fish anti-VHSV antibodies in fish sera and described in Millard and Faisal 2012 were performed. Serum samples were tested for neutralizing antibodies using a 50% plaque neutralization test (50% PNT) with complement addition. Titers are reported as the reciprocal of the highest serum dilution that reduced the number of VHSV plaque-forming units (pfu) by 50% compared to naïve muskellunge sera.

Task 1.6. To determine if VHSV detection is influenced by the disease course.

- Data generated by both lethal and non-lethal sampling was analyzed to compare the use of qPCR on non-lethal samples with using conventional cell culture and nPCR on lethal samples.
- Results were used to determine if the course of the disease and the dose of infection have significant effects on the sensitivity of non-lethal vs. lethal sampling assays.

Objective 2: To compare between lethal and non-lethal (blood, mucus, and uro-fecal swab) samples for the detection of *Rs* following a standardized experimental infection.

Task 2.1: Experimental challenge of chinook salmon with *Renibacterium salmoninarum*.

- Cryopreserved *Rs* (ATCC 33209) was revived on modified kidney disease medium (MKDM), purity verified, and then a single colony was inoculated into a 7 ml aliquot of MKDM broth (x4) and incubated at 15 °C for approximately 7 days. Twenty µL from each of the four broth cultures were then sub-cultured onto trypticase soy agar (TSA) to verify purity, and the remaining broth added to ~1900 ml of fresh MKDM broth and incubated at 15 °C on a stir plate at approximately 50 rpm. After 14 days of incubation, the optical density of the broth was 0.58 using a cell density meter (Biowave CO8000, Denville Inc.). The broth culture was then centrifuged at 4300 rpm for 10 min, supernatant discarded, and bacterial pellet re-suspended in 0.85% saline solution, which was repeated a total of 3 times. The *Rs*-0.85% saline suspension was then divided into three equal volumes while being stirred on a magnetic stir plate and subsamples from each of the three aliquots were serially diluted and utilized for plate counts via drop culture. Each *Rs* aliquot was then added to approximately 3.5 L of autoclaved tank water supplemented with 0.85% saline (total final volume of 4 L) for a final concentration of ~2.3×10^7 cfu ml^{-1}. Each suspension was placed in a sterile 5 gallon bucket that was heavily aerated, to which 42 chinook salmon were added. Each bucket was then covered and fish were immersion exposed for one hour. Fish and the *Rs* suspension were then poured into their empty respective holding tanks and flow through water system was resumed.
- To mimic an acute infection, 75 chinook salmon (25 fish/group in triplicate) were intraperitoneally (i.p.) injected with *Rs*. Cryopreserved *Rs* was revived and purity

verified as described above. Approximately 5-10 colonies were inoculated into a 7 ml aliquot of MKDM broth (x4) and incubated at 15 °C for 7 days. Twenty µL from each of the four broth cultures were then sub-cultured onto TSA and MKDM plates to verify purity, and the remaining broth was added to ~1000ml of fresh MKDM broth and incubated at 15 °C on a stir plate at approximately 50 rpm. After 14 days of incubation, the optical density of the broth was 0.71 using a cell density meter. The broth culture was then centrifuged and washed as described above. The remaining pellet was re-suspended in ~101 ml of sterile saline, for a final concentration of 2.1×10^{10} cfu ml^{-1}. Fish were anesthetized with 100 mg/L of MS-222. Three replicates of 25 fish were injected i.p. with 200 µL of the infection solution. One tank of 25 fish served as a negative control and was injected i.p. with 200 µL of sterile saline. Fish were then revived in fresh water in their respective holding tanks.

Task 2.2: Non-lethal sample collection and detection of *Rs*.

- Mucus was collected with an individual cover slip by gently scraping from the left pectoral fin to the caudal fin. Mucus was then placed into sterile 1.5 ml microcentrifuge tubes and frozen at -80 °C until assayed.
- A portion of the mucus sample was diluted 1:10 (weight:volume) in sterile phosphate-buffered saline, suspended by repeated expulsion through a sterile pipette, and 10 µl was dispensed onto MKDM plates in serial 10-fold dilutions (10^{-1} to 10^{-8}). The inoculated plates were incubated at 15 °C and were examined every week for bacterial growth, for a maximum of 6 weeks. Morphological characteristics of any bacterial growth observed during the 6 week period was recorded, and *Rs* identification was confirmed with nested PCR (nPCR).
- Additionally, a part of the mucus sample was used for DNA extraction and nPCR to evaluate the presence of *Rs*. The DNA was extracted using a Qiagen DNeasy Blood and Tissue Extraction Kit (Qiagen, Inc., Valencia, CA), and was then quantified with the Qubit® Fluorometer (Life Technologies, Grand Island, NY). The DNA was diluted to a 20 ng/µl concentration and used in the nPCR method outlined in Pascho et al. (1998), with minor modifications.
- Lastly, the remaining mucus sample was analyzed by a quantitative enzyme-linked immunosorbent assay (Q-ELISA) for the detection of antigen to *Rs*, according to the general Q-ELISA protocol outlined in Faisal and Eissa (2009). Aliquots of 250 µL of mucus were added to 1.5 ml microcentrifuge tubes containing 250 µL of Phosphate Buffered Saline with Tween-20 (PBS-T20; Sigma) and 5% goat serum (Sigma) and 50 µL of CitriSolv (Fisher Scientific; Pittsburgh, PA). Samples were vortexed for approximately 10 sec, heated at 100 °C for 15 min, and then centrifuged at 14,000 rpm for 10 min. The aqueous supernatant of each sample was used for Q-ELISA testing. The positive-negative cutoff absorbance for the samples was 0.10. Samples that tested positive were assigned the following antigen levels: low (0.10-0.199), medium (0.20-0.999), and high (≥1.00). Each assay included two negative controls (a negative fish tissue sample and a dilution buffer) and two positive controls (a positive fish tissue sample and an *Rs* positive control (Kirkegaard and Perry Laboratories; Gaithersburg, MD).

- Whole blood was also inoculated directly onto MKDM plates, incubated at 15 °C, and observed as described above.
- DNA extractions, nPCR, and Q-ELISA were all performed on serum samples as described above.
- A portion of the uro-fecal sample was inoculated onto MKDM plates in serial 10-fold dilutions, as described above. Additionally, the uro-fecal sample was tested via the Q-ELISA protocol, as well as the nPCR method.
- A gill culture was taken at the time of non-lethal sampling and inoculated directly onto MKDM plates. Cultures were incubated at 15 °C, and examined weekly as described above.

Task 2.3: Lethal sample collection and clinical examination.

- Kidney and spleen samples were processed for *Rs* isolation on MKDM plates in serial 10-fold dilutions, nPCR, and Q-ELISA, all as described above. Additionally, quantitative real-time PCR was performed on the kidney and spleen samples.

Task 2.4: Determination of diagnostic testing pattern in infected fish and association with detectability in non-lethally sampled tissues.

- Diagnostic testing patterns were determined according to Faisal and Eissa (2009) using lethal sample results from all assays to determine the stage of disease for each fish.

Task 2.5: Validation of non-lethal sampling.

- Validation of non-lethal sampling against lethal sampling was determined and results of nonlethal samples were compared to those obtained by lethal sampling.

Objective 3: To compare lethal and non-lethal sampling methods for detecting VHSV and *Rs* in naturally infected fish stocks and to determine if the pathogen/viral load plays a role in non-lethal detection efficacy.

Objective 4: To determine the best statistical approach for relating non-lethal diagnostic tests to prevalence of disease and to determine the appropriate number of fish to sample using non-lethal (imperfect) diagnostic tests to detect the prevalence of a disease in a subpopulation or population of fish.

Task 4.1: Various multivariate, univariate and multiple regression model fitting procedures were performed to determine the best approach for predicting the prevalence of disease using the diagnostic data collected from non-lethal approaches with the corresponding lethal samples.

Task 4.2: Used Bayesian and frequentist statistical approaches (and power analyses) to determine the appropriate sample size for determine the prevalence of disease in a subpopulation of fish using non-lethal diagnostics.

Executive Summary/Abstract for Project:

The use of lethal samples allows the collection of tissues/organs that are preferred by the pathogen, thereby maximizing the likelihood of detecting positively infected individuals. The sacrifice of individuals also permits investigators to conduct

histopathology analysis and visualization of lesions in stained tissue sections, a step that is necessary to identify the pathogen impact on its host. However, lethal procedures are not ideal for threatened/endangered fishes or in fish populations involved in restoration programs. In addition, lethal procedures for disease surveillance in highly valuable broodstock, such as those used in the salmonid conservation programs of the Great Lakes, increase overall costs, reduce broodstock availability, and increase the number of broodstock necessary to satisfy stocking quotas. As such, non-lethal sampling methods have been recommended for the detection of a number of fish disease-causing microbes.

The use of non-lethal procedures has a number of benefits, including reduced sample collection time and the elimination of euthanizing the test subject. The data obtained from these samples would also provide real-time data into the host-pathogen dynamics if sampling could be repeated for quantification of pathogen load and host immune responses. This is critical in that samples collected from fish that are sacrificed often present a momentary window into the host-pathogen interactions.

This study was designed to evaluate non-lethal sampling strategy that will allow the detection of two important diseases that devastate a wide variety of Great Lakes fish species: VHSV and BKD. The new developed sampling methodology coupled with the use of sensitive assays allows the screening of fish with relative ease and with high specificity and sensitivity. Armed with the newly generated information, we used frequentist and Bayesian statistics to determine the likelihood of a disease outcome given a series of non-lethal diagnostic tests and to determine the sample size needed to detect disease prevalence in subpopulations or populations of fish.

Major findings and accomplishments:

I. **Viral Hemorrhagic Septicemia Virus:** VHSV experimental infection to muskellunge was achieved. Clinical signs typical of VHSV developed and their severity and frequency coincided with the administered dose (**Figure I.1a-d**).

High VHSV dose group:

- In the lethal samples from the high virus dose fish group, VHSV was re-isolated and its identity confirmed by PCR. No virus was isolated on the first day post-infection (p.i.), yet VHSV was isolated from 100% of infected fish samples on days 4, 6, and 7 p.i. with both cell culture and PCR (**Figure I.2a**).
- Mucus samples of the high virus dose group exhibited very comparable results to those observed in the lethal samples; i.e., all samples from day 1 p.i. were negative on cell culture and PCR while 83% of the samples collected on days 4, 6, and 7 p.i. were VHSV positive on cell culture. 100% of the mucus samples from days 4, 6, and 7 were VHSV-positive by PCR or real-time PCR, including those that tested negative on cell culture (**Figure I.2a**).
- Uro-fecal samples from fish infected with the high VHSV dose were negative on 1, 4, 6, and 7 days p.i. with both cell culture and PCR. However, one sample collected on day 4 p.i. was VHSV positive through real-time PCR and one sample collected on day 7 p.i. was VHSV positive based on plaque formation (**Figure I.2a**).

- The virus was detected in 60% of sera collected from fish in the high dose group. Isolation occurred as early as 2 days p.i. and was isolated from the majority of sera from days 3 (87%), 4 (93%), and 7 (87%) p.i. (**Figure I.3**).

Intermediate VHSV dose group:

- In the lethal samples of the medium dose fish group, VHSV was not isolated from any individuals on day 1 p.i. On days 3, 10, and 28 p.i., VHSV was isolated from 63% of individuals on cell culture. An additional 11% of the lethal samples that were negative on cell culture from days 3, 10, and 28 p.i. were positive for VHSV by PCR or real-time PCR (**Figure I.2b**).
- Mucus samples from the medium virus dose group were 100% negative on day 1 p.i.; however, cell culture results yielded 30% positive VHSV samples during days 3, 10, and 28 p.i. An additional 15% of samples from days 3, 10, and 28 p.i. that were negative on cell culture were positive using PCR or real-time PCR. (**Figure I.2b**).
- Of all uro-fecal samples from fish infected with the medium VHSV dose, 3% were positive for VHSV on cell culture, which was detected on fish sampled from day 3; all uro-fecal samples collected on days 1, 10, and 28 p.i. produced negative cell culture results. Furthermore, 14% of all uro-fecal samples tested positive for VHSV through the use of PCR or real-time PCR (**Figure I.2b**).
- Virus was isolated from 35% of fish sera collected from fish infected with the medium dose. VHSV was detectable in sera by 3 days p.i. and was last detected 21 days p.i. The highest numbers of positive sera were collected on days 7, 10, and 14 p.i. (**Figure I.3**).

Low VHSV dose group:

- In the lethal samples of the low virus dose fish group, VHSV was again negative in 100% of those collected on day 1 p.i. On days 21, 42, and 56 p.i., VHSV was isolated by cell culture from 40% of individuals. Additionally, 17% of samples from those days that were negative by cell culture were found positive by PCR or real-time PCR (**Figure I.2c**).
- Mucus samples from the low virus dose group were again 100% negative from day 1 p.i. On days 21, 42, and 56 p.i., 13% were positive by cell culture. Of those that were negative by cell culture, 12% were affirmed positive by PCR or real-time PCR (**Figure I.2c**).
- Uro-fecal samples from fish infected with the low dose of VHSV were 98% negative by cell culture on days 1, 21, 42, and 56 p.i.; however, 10% of those individuals that were negative by cell culture were detected positive through the use of PCR or real-time PCR (**Figure I.2c**).
- In the low dose group, 7% of fish had VHSV in their sera. The virus was not detected until later during the disease course (starting at 28 days p.i.) and was last detected from sera collected at 42 days p.i. (**Figure I.3**).

Negative control group:

- All kidney, spleen, mucus, and uro-fecal samples collected from the negative control group tested negative for VHSV by cell culture, and was confirmed by both PCR and real-time PCR (**Figure I.2a**).

- VHSV was not isolated from any fish sera of the negative control group. (**Figure I.3**).

A clear correlation could be drawn between the detection of VHSV in mucus and in internal organs. The correlation coefficient ranged from 0.54 to 1.0. This is an important milestone that will facilitate wide scale VHSV surveys, lower the costs, and is ideal for preserving valuable broodstocks such as lake sturgeon.

Detection of anti-VHSV antibodies in sera of fish collected in endemic waters:

- Sera from a total of 48 negative control fish were tested individually. The majority of these fish had titers of <20. Neutralization against VHSV was detected in sera of some fish from each dose group **(Figure I.4).** Sera from fish infected with a low dose of virus had the highest percentage of individuals with neutralization titers (27%). It is possible that this is in part due to the fact that these fish were sampled later into the disease course than the other two groups. The highest number of responding low-dose fish were sampled on days 42 and 49 p.i. The highest 50% PNT titers (1280 to 2560) were detected on 14 and 28 days p.i.
- For muskellunge infected with medium and high doses, 8% and 23% of sera (for these groups respectively) were positive by the 50% PNT. The highest percentage of neutralization activity was detectable on the last sampling day for the high dose group. It is interesting to note that early in the disease course, most sera with neutralizing titers were also VHSV positive. Conversely, most sera from surviving fish sampled later in the disease course were negative for the virus. This could indicate that the antibodies successfully limited the virus in these fish (**Figure I.4**).
- Both lethal and non-lethal samples were collected from Lake St. Clair muskellunge during May-June 2010 and 2011. All of the 41 fish tested were negative for VHSV. When sera from these fish were tested by the 50% PNT, however, 83% exhibited neutralization against the virus, with titers ranging from 160 to 327,680 (**Figure I.5**). These results indicate that in a VHSV endemic water body, fish that have been exposed in the past to the virus can be detected by antibody-based methods. *Therefore, for surveillance in VHSV-positive water bodies, there is no need to do further lethal testing of the population.*

II. *Renibacterium salmoninarum*: Experimental infection was successful and clinical signs characteristic of bacterial kidney disease (BKD) developed (**Figure II.1**). Throughout the study, low to high loads of *Rs* were consistently isolated from infected fish. There was a greater amount of detection of *Rs* in the injection challenge (100%) than the immersion challenge (87%); however, this is not surprising since, by injection, natural barriers are overcome and infection is ensured. The detection of *Rs* was much greater in the lethal samples (kidney and spleen; 73%) compared to the non-lethal samples (mucus, blood, serum, and uro-fecal samples; 36%) in the immersion challenge (**Figure II.2**), while in the injection challenge, the percentage of *Rs* detected in the lethal samples was equivalent to the detection in the non-lethal samples (100%; **Figure II.2**).

Immersion challenge:

- Overall, the percentage of *Rs* detected during the immersion challenge varied by sampling period. Sampling periods were at 1, 21, 42, 63, 84, and 105 days p.i. The prevalence of *Rs* detected in the non-lethal samples ranged from 100% infected at day 1 p.i., to only 24% of fish infected at day 21 p.i., which then decreased to 0% infected at day 42 p.i., then increased to 67% infected at day 63 p.i., decreased again to 6% infected at day 84 p.i., and finally decreased to 0% infected at day 105 p.i. (**Figure II.3**).
- The detection of *Rs* in the lethal samples from the immersion challenge demonstrated a gradually increasing trend throughout the study, with 33% of fish infected at day 1 p.i., 67% infected at day 21 p.i., 91% infected at day 63 p.i., decreased slightly to 82% infected at day 84 p.i., and finally increased to 100% of the fish infected at day 105 p.i. (**Figure II.3**).
- There was also variation in the detection of *Rs* in the different types of non-lethal tissues (i.e., mucus, blood, serum, and uro-fecal samples) in the immersion challenge (**Figure II.5**). The mucus samples were the most consistently infected non-lethal tissue, having been found to be positive for *Rs* in 3 of the 6 sampling periods (**Figure II.5**). The prevalence of mucus samples positive for *Rs* was 24% at days 1 and 21 p.i., and 10% at day 63 p.i. Additionally, the prevalence of *Rs* in the uro-fecal samples was as high as 100% at day 1 p.i. of the immersion challenge and 67% at day 63 p.i. (**Figure II.5**). The blood samples yielded very little *Rs* isolation, with a prevalence of only 5% infected at day 1 p.i. and 6% infected at day 84 p.i. (**Figure II.5**). None of the serum samples were positive for *Rs* in the immersion challenge.

<u>Injection challenge:</u>

- Interestingly, in the injection challenge, 100% of the non-lethal and lethal samples were positive for *Rs* throughout the duration of both challenges at each sampling period (**Figure II.4**). Sampling periods were at 1, 8, 15, 22, and 29 days p.i. It is clearly evident that there was an active *Rs* infection in the fish throughout the study. As was expected, the rate of infection in the fish from the immersion challenge was more gradual, whereas the infected fish from the injection challenge were highly infected throughout the entire experiment.
- The detection of *Rs* was consistently higher in the injection challenge; yet, the prevalence of the bacterium still varied depending upon the type of non-lethal tissue. The percentage of positive mucus samples increased steadily throughout the challenge, from 0% infected at day 1 p.i., 13% infected at day 8 p.i., 87% at day 15 p.i., and 100% infected at days 22 and 29 p.i. (**Figure II.6**).
- In the injection challenge, the remaining non-lethal tissues (blood, serum, and uro-fecal samples) all tested positive for *Rs* in each of the five sampling periods (**Figure II.6**). The prevalence of blood samples that were positive for *Rs* during the injection challenge ranged from 93% infected at day 1 p.i., 73% infected at day 8 p.i., 100% infected at day 15 p.i., 73% infected at day 22, and finally to 67% infected at day 29 p.i. (**Figure II.6**). The prevalence of *Rs* in the serum was relatively high at a rate of 100% infected during each sampling period, with the exception of day 29 p.i., when it decreased to 67% infected (**Figure II.6**). The prevalence of *Rs* in the uro-fecal samples was similar to the serum, with 100% of the fish infected for each of the sampling periods, except for day 20 p.i. when it

decreased to 33% infected (**Figure II.6**). This may have been indicative of the fish starting to clear the pathogen from their bodies.

Testing Methods:

- In addition to evaluating the presence of *Rs* in the lethal and non-lethal fish tissue, the effectiveness of the different testing methods was also examined. There are several accepted methods for the detection of *Rs* that were utilized throughout this study: traditional culture on selective media, Q-ELISA, nPCR, and qPCR. It has been demonstrated previously that discrepancies in the testing methods used to detect *Rs* can occur and distinct testing patterns can emerge (Faisal and Eissa 2009).
- In both of the challenges in this study, the testing patterns varied for the detection of *Rs*. Interestingly, the traditional culture method detected more *Rs*-positive samples in the non-lethal samples (8%) compared to the lethal samples (4%) in the immersion challenge (**Figure II.7**). Additionally, similar results were seen for the injection challenge, where 76% of the non-lethal samples were positive for *Rs*, and only 52% of the lethal samples were positive for *Rs* by culture.
- The Q-ELISA method detected a greater prevalence of lethal samples that were positive for *Rs* (71%) than non-lethal samples (10%) in the immersion challenge (**Figure II.8**). While in the injection challenge, the Q-ELISA method detected fairly similar prevalences of *Rs* in the lethal (100%) and non-lethal (98%) samples (**Figure II.8**).
- Interestingly, during the immersion challenge, the nPCR method detected a higher prevalence of *Rs* in the non-lethal samples (30%) than the lethal samples (0**%**; **Figure II.9**). Yet, the level of detection of *Rs* by nPCR in the injection challenge was the same for the non-lethal (100%) and lethal samples (100%; **Figure II.9**). Quantitative-PCR was only performed on the lethal samples, and the results were the same as the nPCR results. Also, in the immersion challenge, most samples that tested positive for *Rs* did so by the Q-ELISA method. Whereas in the injection challenge, most of the *Rs*-positive samples were detected equally by Q-ELISA and nPCR, followed closely by culture. Therefore, it appears that in an infection similar to the injection challenge, Q-ELISA and nPCR are similarly effective in the detection of *Rs*. However, Q-ELISA by itself shows better potential as a detection tool during an infection that mimics the immersion infection.
- The results of this study agree with the findings of previous research, demonstrating that there is variation in the detection of *Rs* in the different testing methods. However, the assays used in this study are all detecting different aspects of the bacterium. The Q-ELISA assay mainly targets *Rs*-specific proteins and antigen, while PCR and culture detect the actual bacterium. Yet while culture requires the presence of live bacteria to grow on a medium, PCR only needs the DNA from the bacteria (alive or dead) to detect the pathogen. Therefore, it is expected for there to be some variation in the results from the different assays. Additionally, this variation in the assay results demonstrated that there were certain testing patterns, as noted in previous studies (**Figure II.10**). Pattern 1 represented fish that were positive by nPCR only, while Pattern 2 represented fish that were positive by nPCR and culture. Fish that were positive by all three

testing methods fell into Pattern 3 and fish that were positive by nPCR and Q-ELISA only were in Pattern 4. Pattern 5 represented fish that were only positive by Q-ELISA, and fish that were completely negative for *Rs* were in Pattern 6. Pattern 7 represented fish that were positive by culture only, and Pattern 8 represented fish that were positive by culture and by Q-ELISA.

- Overall, from both immersion and infection challenge results combined, the majority of fish whose non-lethal tissue samples produced positive *Rs* results from any assay were most likely to yield positive results from all 3 assays; i.e., Pattern 3 (28%; **Figure II.10**). Other non-lethal samples tested for *Rs* were in Pattern 1 (13%), Pattern 2 (1%), Pattern 4 (13%), Pattern 6 (42%), and Pattern 7 (3%; **Figure II.10**).
- In the lethal tissues sampled from both the immersion and infection challenge groups, most of the *Rs*-positive samples were in Pattern 5 (46%; **Figure II.10**), which was only Q-ELISA positive. Additionally, lethal samples tested for *Rs* were also in Pattern 3 (19%), Pattern 4 (16%), Pattern 6 (17%), Pattern 7 (1%), and Pattern 8 (2%; **Figure II.10**).
- From non-lethal samples collected from the immersion challenge only, the majority of the fish that tested positive for *Rs* were positive by only nPCR, which was Pattern 1 (19%; **Figure II.11**). The remaining non-lethal samples from the immersion challenge were in Pattern 2 (2%), Pattern 3 (1%), Pattern 4 (9%), Pattern 6 (64%), and Pattern 7 (5%; **Figure II.11**).
- The majority of the positive lethal samples from the immersion challenge were in Pattern 5, which was positive by Q-ELISA only (69%; **Figure II.11**). The remaining lethal samples from the immersion challenge were in Pattern 5 (69%), Pattern 6 (27%), Pattern 7 (2%), and Pattern 8 (3%; **Figure II.11**).
- However, the injection challenge yielded different results. The majority of non-lethal samples that were positive for *Rs* were positive by all three assays, which was Pattern 3 (78%; **Figure II.12**). The remaining non-lethal samples from the injection challenge were in Pattern 1 (2%) and Pattern 4 (21%; **Figure II.12**).
- The majority of the positive lethal samples from the injection challenge were also in Pattern 3 (52%; **Figure II.12**). The remaining lethal samples from the injection challenge were in Pattern 4 (44%) and Pattern 5 (3%).

Management implications of our work:

1. The use of non-lethal samples allows cost-effective surveillance and monitoring of two serious diseases with high specificity.
2. Knowledge of the disease dynamics and pathogen trafficking will allow managers to identify sources and reservoirs of the infections and therefore enable them to eliminate these sources.
3. At a higher dose of infection, it is possible for non-lethal sampling to detect levels of pathogens in infected fish tissues similar to the lethal sampling.
4. From a management perspective, non-lethal sampling would be beneficial for state hatcheries that routinely sacrifice fish for annual health inspections. The ability to sample non-lethally would ensure that the hatcheries would not need to sacrifice any of their important fish stocks.

5. Also, the use of non-lethal sampling could potentially reduce the cost, time, and manpower that are currently needed for lethal sampling.
6. The use of serological assays allows infected populations to be identified even in the absence of pathogen detection.

Additional restoration work needed and/or areas for future research:
There is a need to continue the research into the validity of non-lethal sampling and the numbers of fish needed for sampling determined. There is a need to start applying non-lethal sampling into surveillance

Geographic region project occurred in or effects:
While this project addresses two important fish diseases prevalent in the Great Lakes basin, the obtained results can be applied to other fish diseases and geographic locations.

List of presentations delivered, reports and peer-reviewed papers completed or in-progress:
Millard, E. and M. Faisal. In press. Heterogeneity in Levels of Serum Neutralizing Antibodies Against Viral Hemorrhagic Septicemia Virus (Genotype IVb) among Fish Species in Lake St. Clair, Michigan, USA. Journal of Wildlife Diseases.

Millard, E. and M. Faisal. Serological response of Great Lakes fishes to Viral Hemorrhagic Septicemia Virus. Presented at the 36th Annual Eastern Fish Health Workshop, Charleston, South Carolina March 28-April 1, 2011.

Millard, E., M. Faisal, and S. LaPatra: Presence of Serum Neutralizing Antibodies Against Viral Hemorrhagic Septicemia Virus (Genotype IVb) in Fish Residing in Lake St. Clair, Michigan. May 24-28, 2010. Eastern Fish Health Workshop in Shepardstown, West Virginia.

Schulz, C. and M. Faisal. The Decline of Bacterial Kidney Disease in *Oncorhynchus* spp. from Michigan. Presented at the 36th Annual Eastern Fish Health Workshop, Charleston, South Carolina March 28-April 1, 2011.

Throckmorton, E., R. Kim, A. Peters, and M. Faisal. Potential value of non-lethal sampling for VHSV (genotype IVb). Presented at the 36th Annual Eastern Fish Health Workshop, Charleston, South Carolina March 28-April 1, 2011.

Schulz, C. and M. Faisal. In progress. The implications of using non-lethal sampling techniques for the detection of *Renibacterium salmoninarum*, the causative agent of Bacterial Kidney Disease in Chinook salmon (*Oncorhynchus tshawytscha*).

References cited:
Millard, E. and M. Faisal. 2012. Development of neutralizing antibody responses in muskellunge, *Esox masquinongy* (Mitchill), experimentally exposed to viral haemorrhagic septicaemia virus (genotype IVb). Journal of Fish Diseases 35: 11-18.

Faisal, M. and A. E. Eissa. 2009. Diagnostic testing patterns of *Renibacterium salmoninarum* in spawning salmonid stocks in Michigan. Journal of Wildlife Diseases 45(2): 447-456.

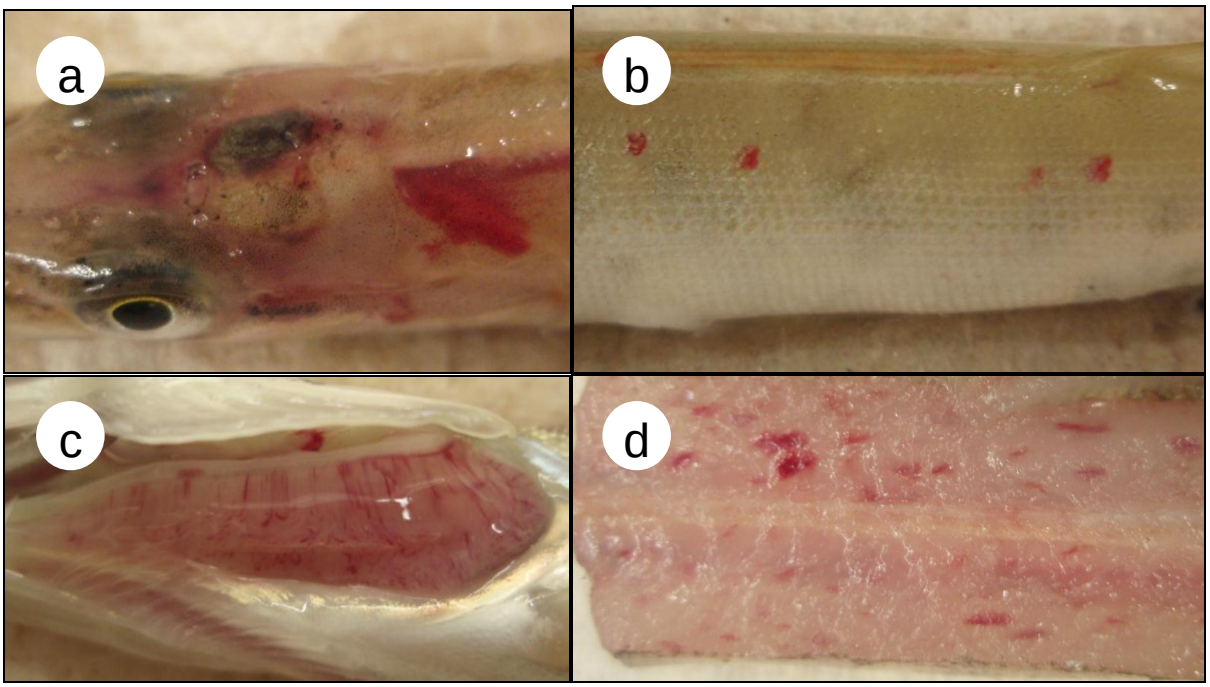
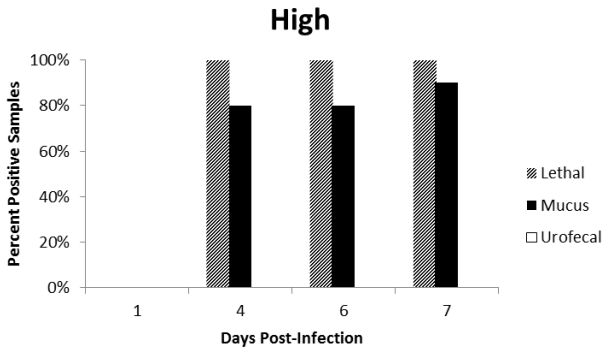
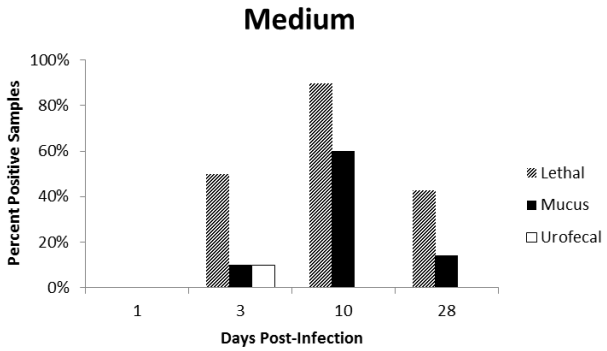


Figure I.1. Clinical signs of muskellunge experimentally infected by immersion with viral hemorrhagic septicemia virus genotype IVb. (a) Ecchymotic hemorrhaging at the nuchal region and skin; (b) dermal petechial hemorrhages; (c) severely pale gills; (d) severe intramuscular hemorrhages.

a.



b.



c.

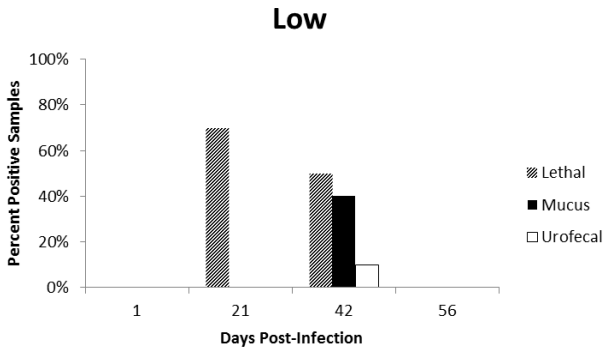


Figure I.2. Viral hemorrhagic septicemia virus isolation by cell culture from lethal and non-lethal sampling methods of muskellunge experimentally infected by immersion with three different virus doses (high, medium, and low). All negative control group fish were confirmed negative by all assays.

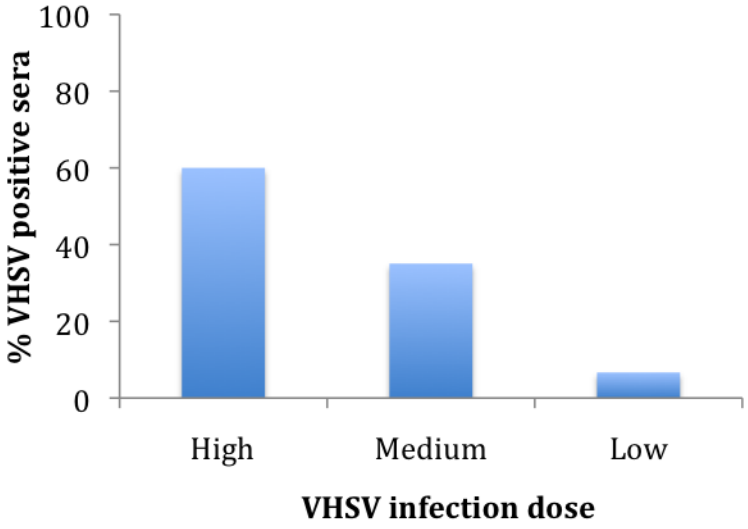


Figure I.3. Percent (%) of viral hemorrhagic septicemia positive (VHSV) muskellunge serum samples by cell culture.

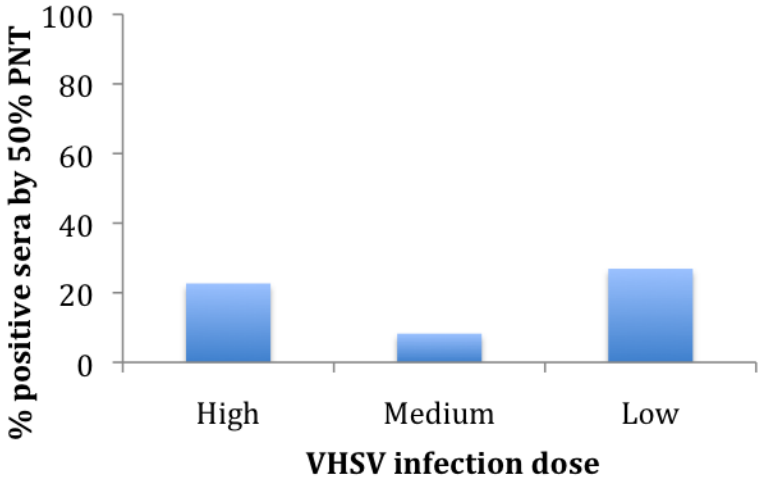


Figure I.4. Percent (%) of viral hemorrhagic septicemia virus positive muskellunge serum samples by 50% plaque neutralization test (50%PNT). Results for each dose are reported as the number of fish with 50%PNT titers ≥20 out of the number of fish tested.

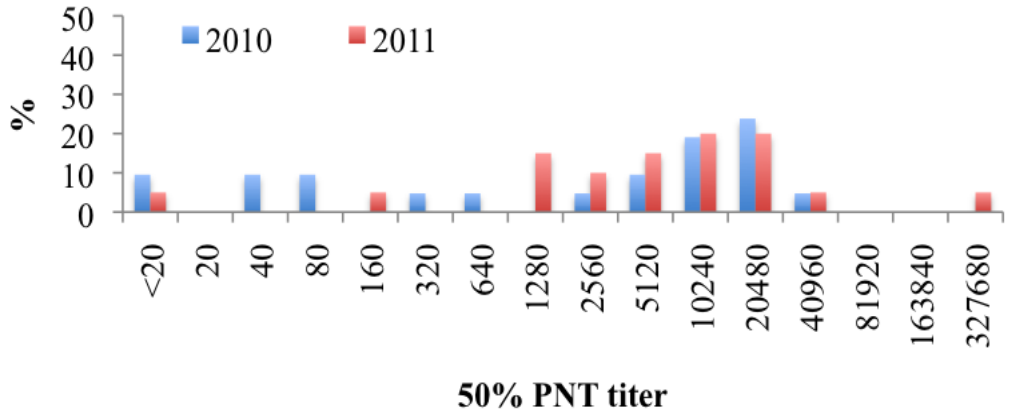


Figure I.5. Distribution of neutralization titers against viral hemorrhagic septicemia virus (VHSV) in sera from Lake St. Clair, Michigan muskellunge sampled in 2010 and 2011. For wild fish sera, titers of ≥160 are considered positive for VHSV-neutralizing antibodies.

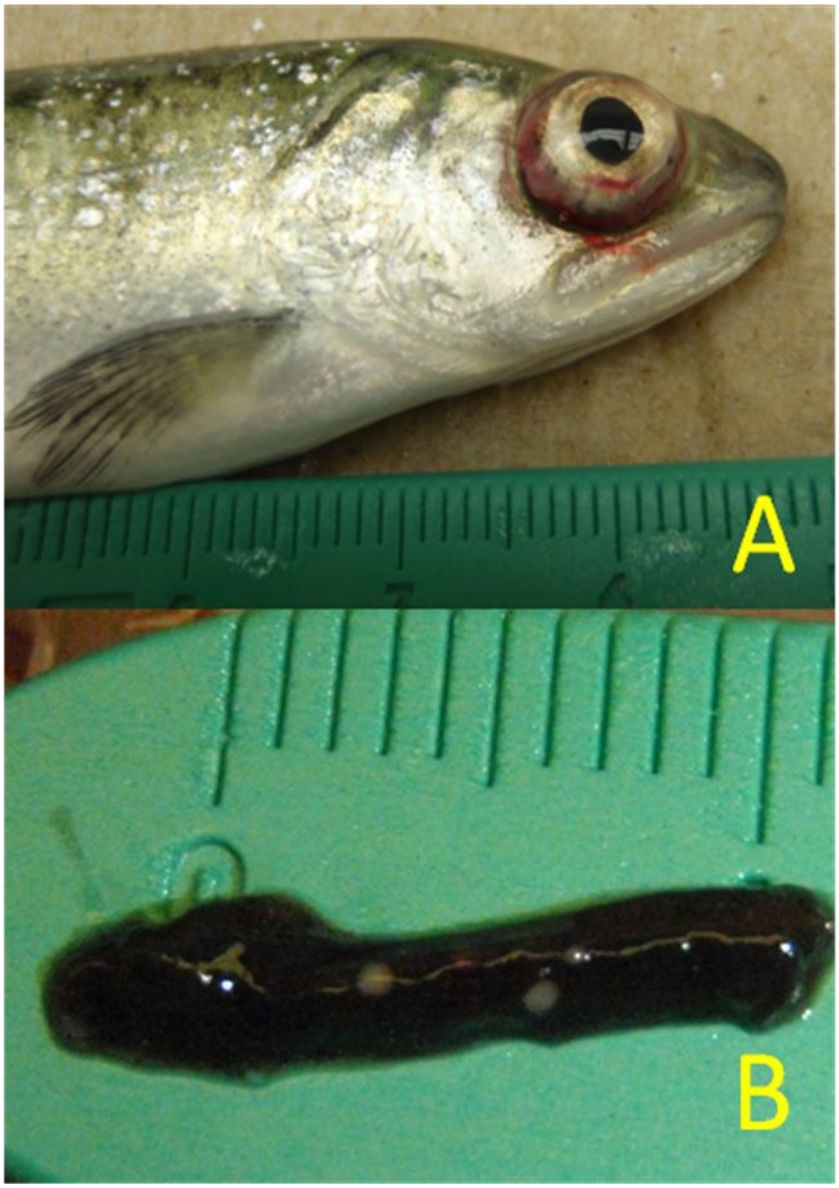


Figure II.1. Chinook salmon experimentally infected with *Renibacterium salmoninarum.* Notice the exophthalmia (A) and whitish nodules of granulomatous tissues in the kidneys (B).

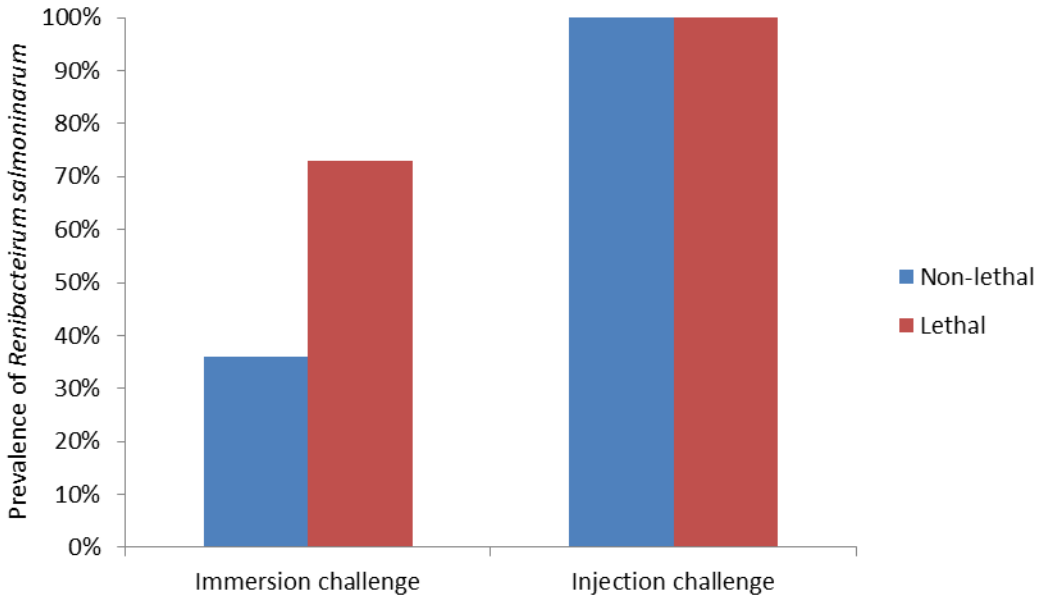


Figure II.2. The prevalence of *Renibacterium salmoninarum* infection in non-lethal and lethal tissue samples collected from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via immersion and intraperitoneal injection.

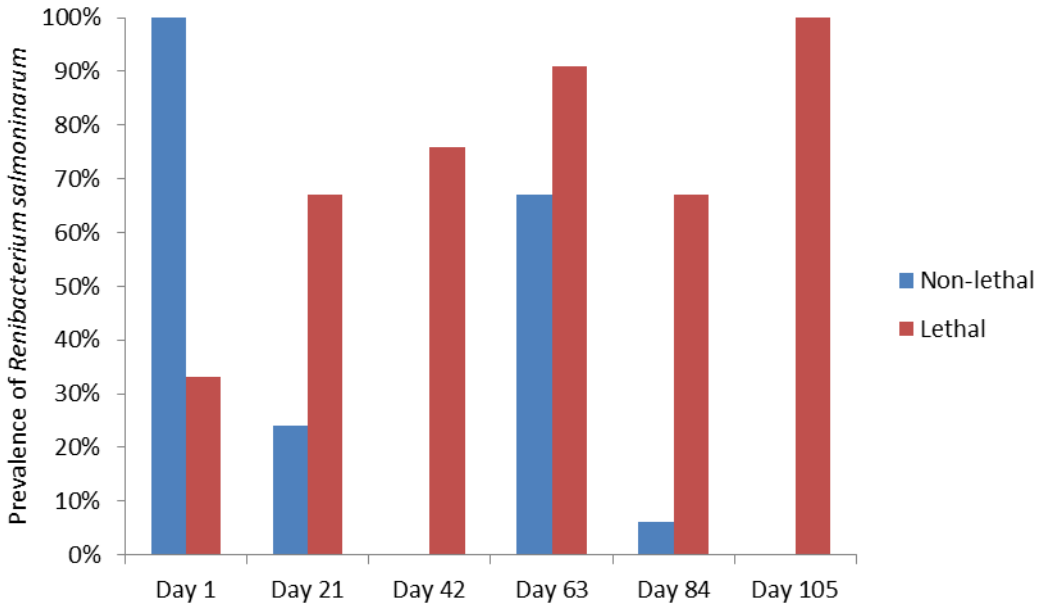


Figure II.3. The prevalence of *Renibacterium salmoninarum* infection in non-lethal and lethal tissue samples from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via immersion for each sampling period.

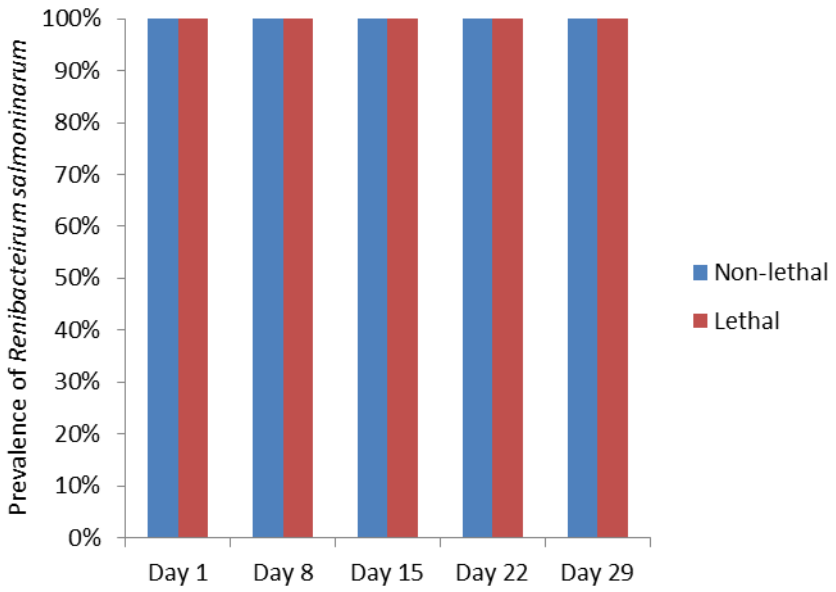


Figure II.4. The prevalence of *Renibacterium salmoninarum* infection in non-lethal and lethal tissue samples from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via intraperitoneal injection for each sampling period.

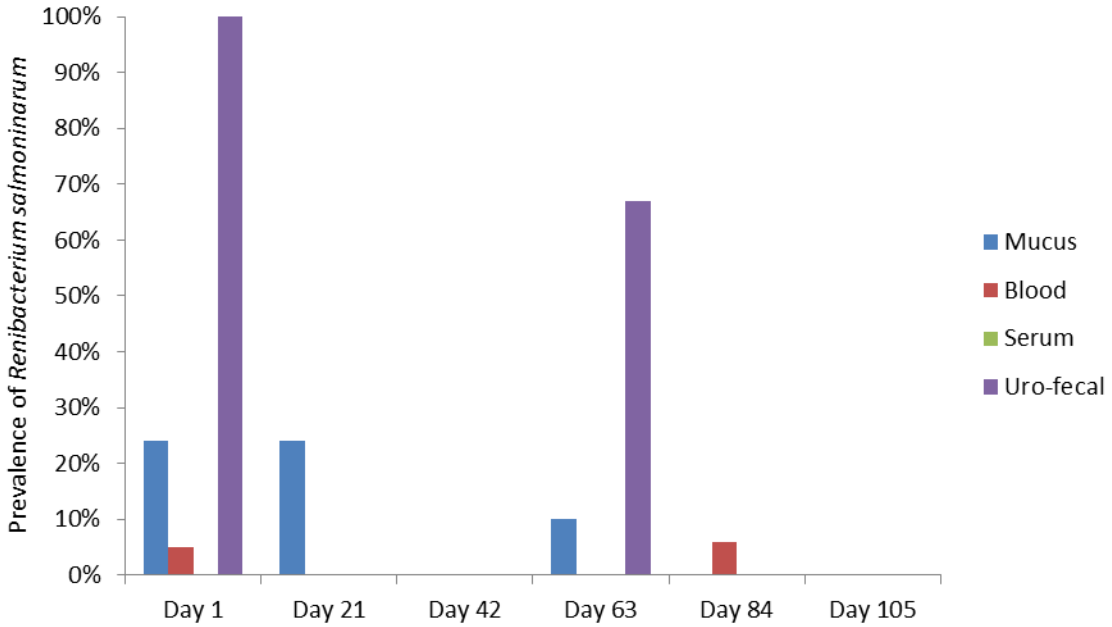


Figure II.5. The prevalence of *Renibacterium salmoninarum* infection in non-lethal tissue samples (mucus, blood, serum, and uro-fecal) from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via immersion for each sampling period.

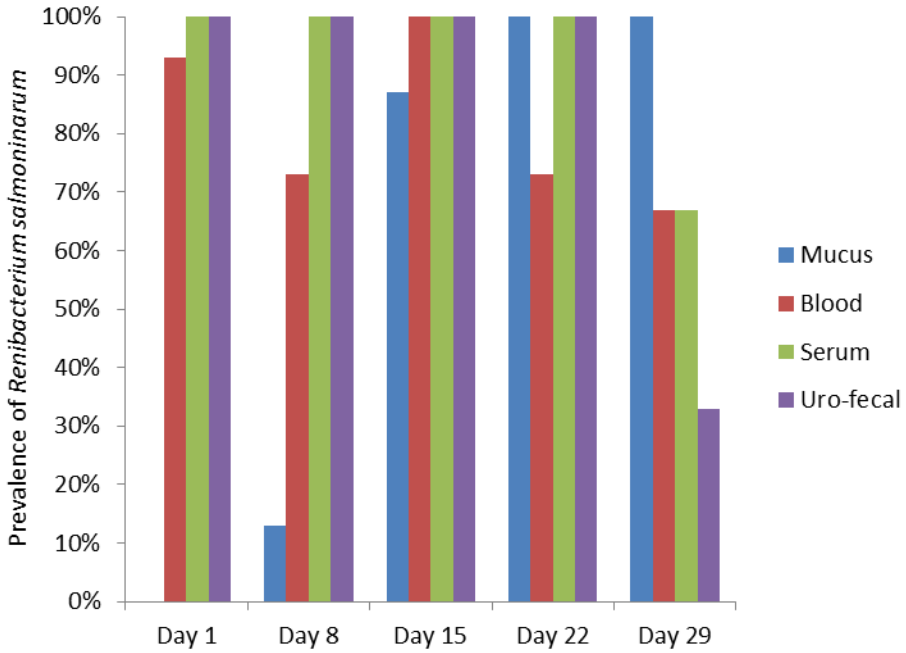


Figure II.6. The prevalence of *Renibacterium salmoninarum* infection in non-lethal tissue samples (mucus, blood, serum, and uro-fecal) from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via intraperitoneal injection for each sampling period.

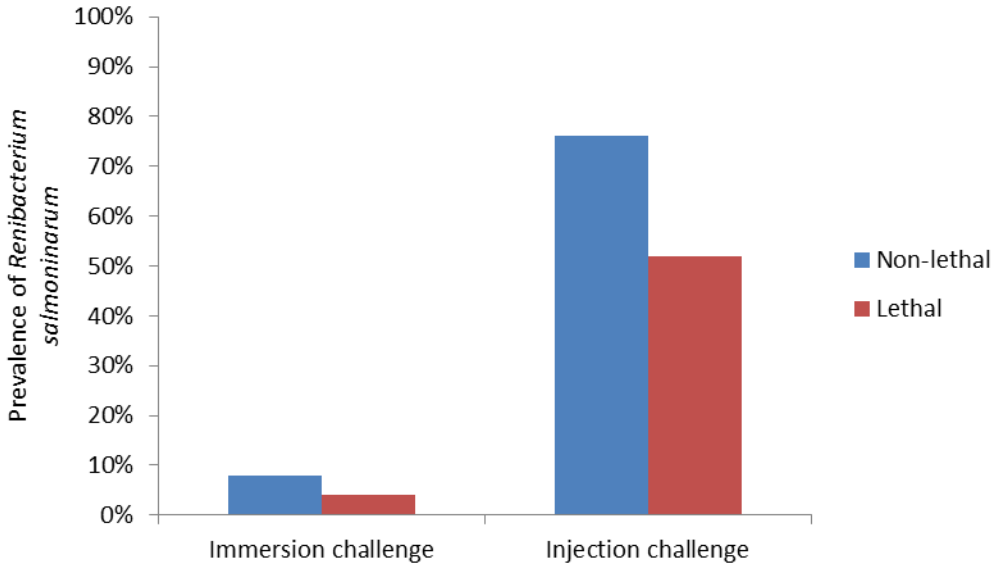


Figure II.7. The prevalence of *Renibacterium salmoninarum* infection in non-lethal and lethal tissue samples from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via immersion and intraperitoneal injection that were tested by the culture method.

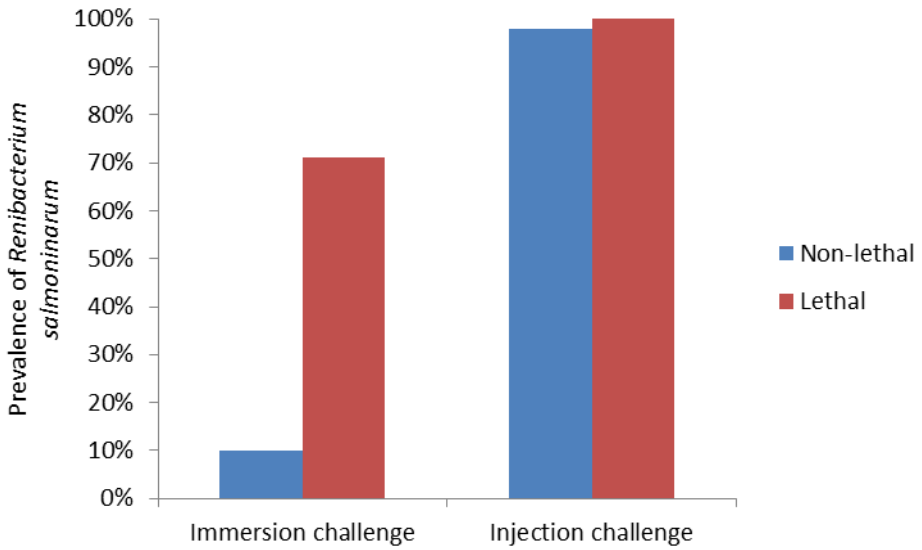


Figure II.8. The prevalence of *Renibacterium salmoninarum* infection in non-lethal and lethal tissue samples from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via immersion and intraperitoneal injection that were tested by the quantitative enzyme-linked immunosorbent assay (Q-ELISA).

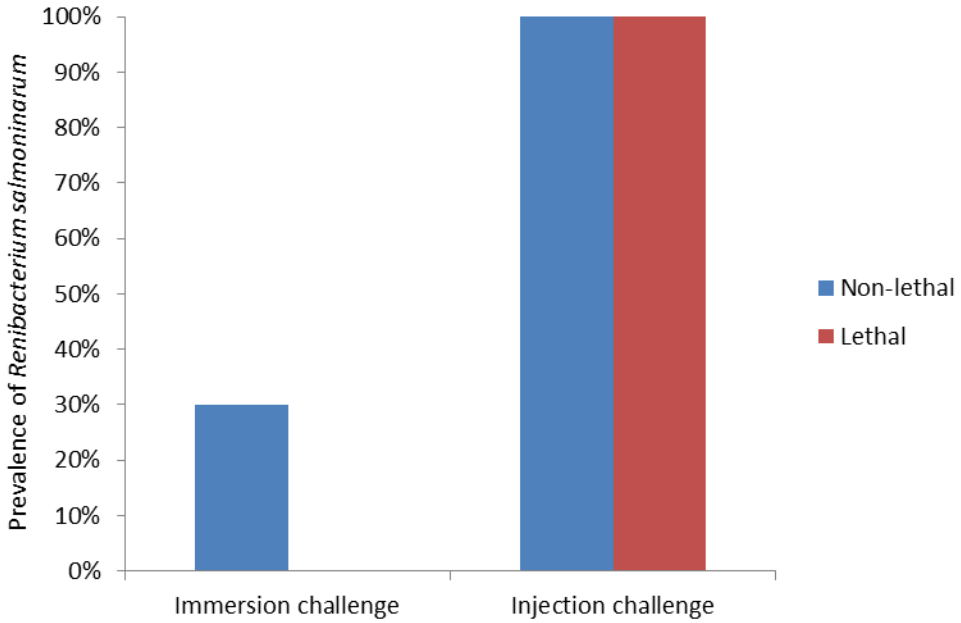


Figure II.9. The prevalence of *Renibacterium salmoninarum* infection in non-lethal and lethal tissue samples from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via immersion and intraperitoneal injection that were tested by the nested Polymerase Chain Reaction (nPCR) assay.

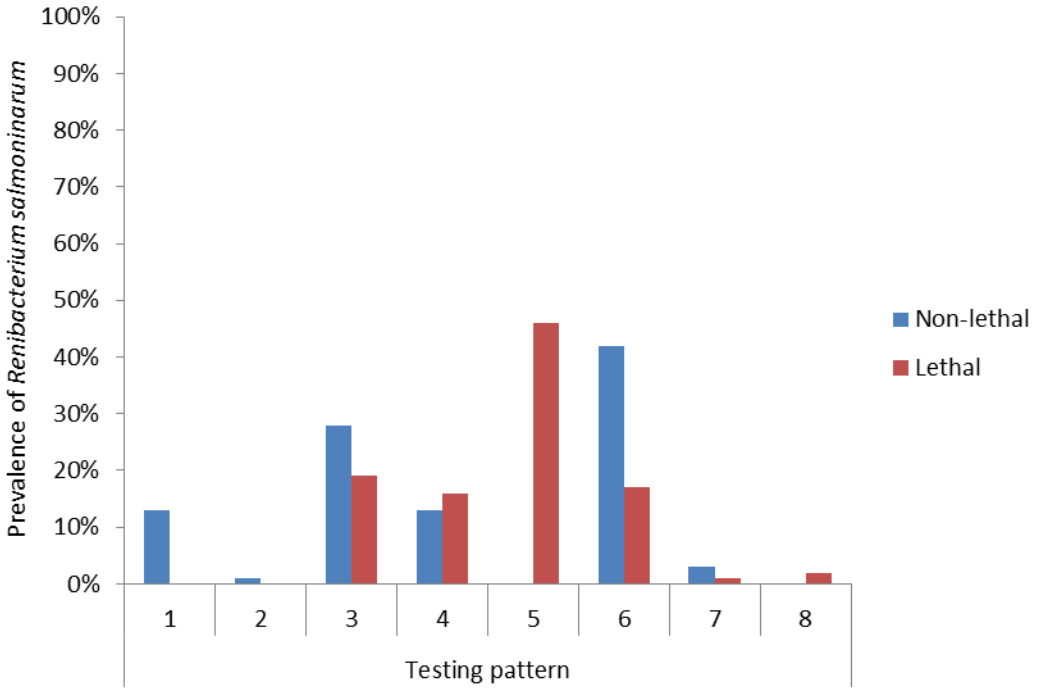


Figure II.10. The prevalence of *Renibacterium salmoninarum* infection in non-lethal and lethal tissue samples from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via immersion and intraperitoneal injection, which were classified into 8 diagnostic testing patterns.

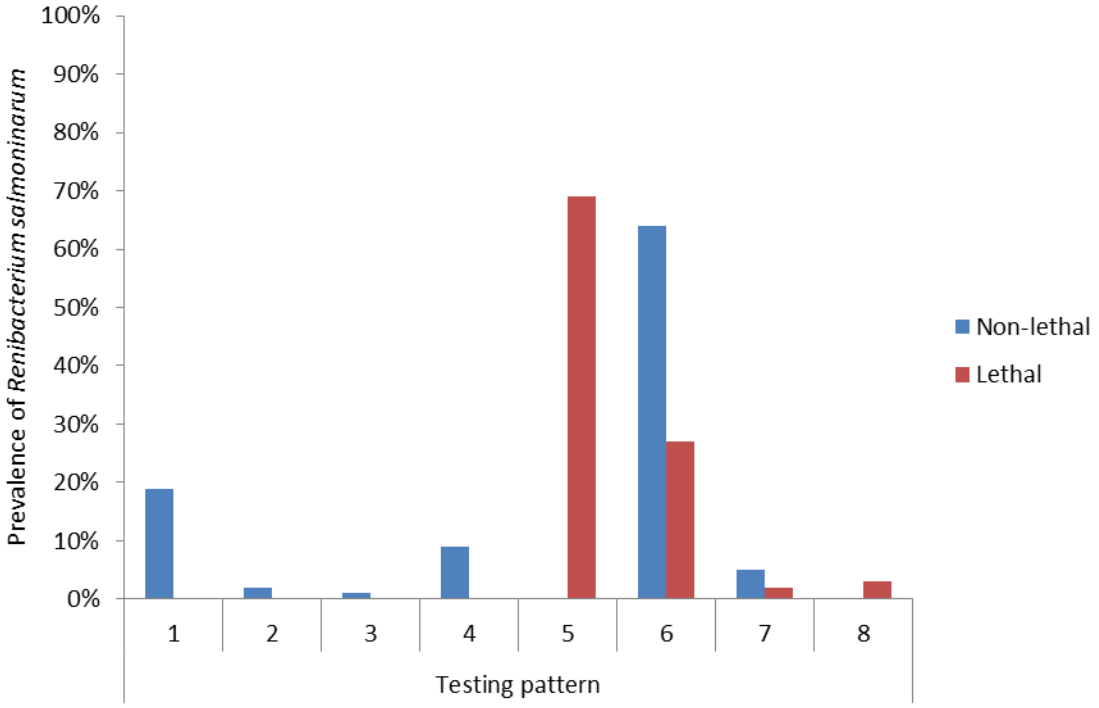


Figure II.11. The prevalence of *Renibacterium salmoninarum* infection in non-lethal and lethal tissue samples from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via immersion, which were classified into 8 diagnostic testing patterns.

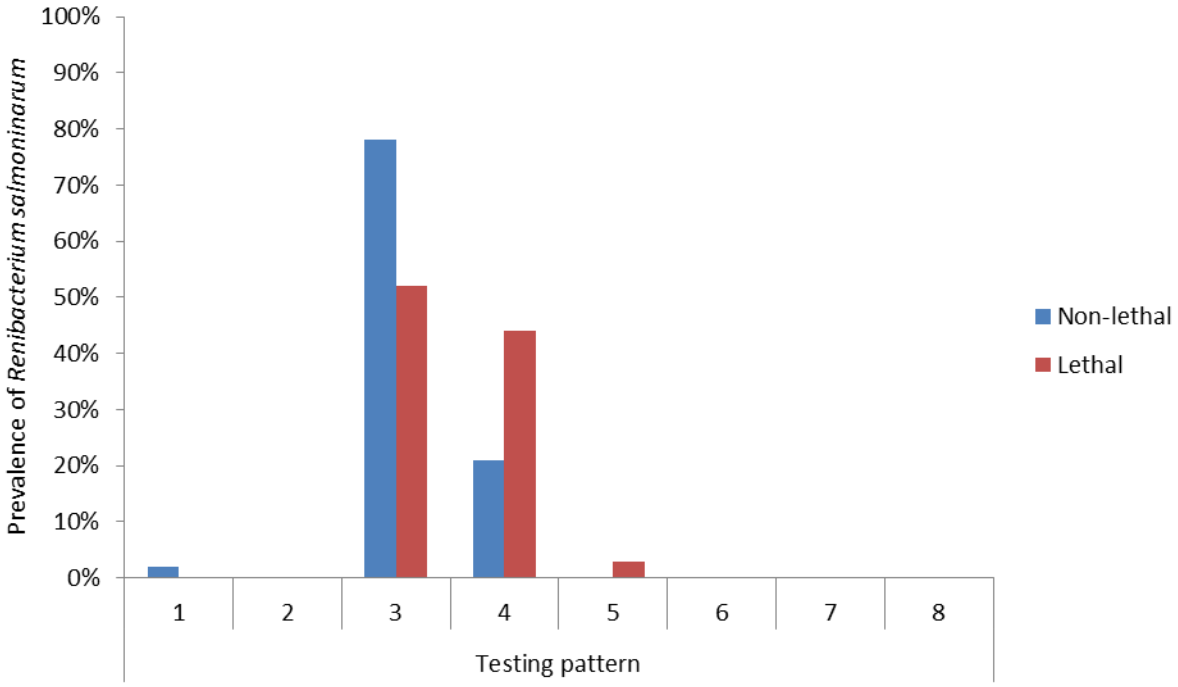


Figure II.12. The prevalence of *Renibacterium salmoninarum* infection in non-lethal and lethal tissue samples from experimentally challenged chinook salmon (*Oncorhynchus tshawytscha*) via intraperitoneal injection, which were classified into 8 diagnostic testing patterns.