

# **Comparative Study of the Sensitivity of Bacterial Culture of *Aeromonas salmonicida* from Individual and Five-fish pool Samples from Naturally Infected Lahanton Cutthroat Trout**

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## **Introduction**

The question of bacterial culture sensitivity for individual versus pooled samples was discussed at the 2001 Fish Health Biologist Meeting in Tucson, AZ, as procedures for the USFWS Handbook were being developed. The USFWS Handbook strives to conform with methods specified by the Office International des Epizooties (OIE 2000) and the American Fisheries Society (AFS) Blue Book (Thoesen, 1996), however these two methodologies differed. The OIE method specifies up to 5-fish pools of target tissue, while the Bluebook recommended the use of inoculating loops to sample individual fish for bacteriological testing.

A comparative study of the two methodologies most commonly used for detection of bacteria was suggested as a means to evaluate the sample method sensitivity and determine if either method would preclude a positive test result during a fish health inspection. The Ca-Nv Fish Health Center volunteered to conduct a comparative study to determine if bacterial culture sensitivity was negatively affected by pooling kidney tissues during sample collection from adult broodstock known to be infected with *Aeromonas salmonicida*. It is important to utilize naturally infected fish because they more closely represent the range of host and environmental variables present in a fish population; this range of variables has as a major impact on the accuracy of the test-result interpretation (OIE, 2000). The study also addressed the effects of delayed inoculation of kidney tissue onto growth media to determine if assay sensitivity was altered when samples were collected in the "field" versus inoculated at a later time in the laboratory.

The study was conducted using Pyramid Lake Lahontan cutthroat trout broodstock which were known to have an incidence of infection with *Aeromonas salmonicida* ranging from 13-50% during routine testing over the past 11 years. Fish were tested in April 2001 during the peak of the spawning season. Thirty male and females were randomly selected to sample for bacteriology. Kidney tissue was collected aseptically, individual fish were inoculated immediately onto BHIA plates, and fish identification tracked to subsequently pool and/or stored tissue for additional tests at 24 hours.

The main objective of the study was to compare culture sensitivity, the ability to detect *Aeromonas salmonicida* cultured on Brain Heart Infusion Agar, of individual and pooled kidney samples. With some additional work, the study also addressed 3 variables that may affect culture sensitivity during routine field collections;

- the size of the tissue used to inoculate the growth medium,
- the period of time from initial collection of tissue to inoculation onto the growth medium,
- the effects of using the viral sample for bacteriology (includes a centrifugation step and use of the kidney pellet to inoculate bacteriological growth medium, following virology processing).

## Study Summary

**Objective #1 - Evaluate two standard inoculation methods, 10ul inoculating loop and 1-cm<sup>2</sup> piece of kidney tissue, to determine if tissue size affects detection sensitivity of *Aeromonas salmonicida* cultured on BHIA.**

In general, culture sensitivity increased when:

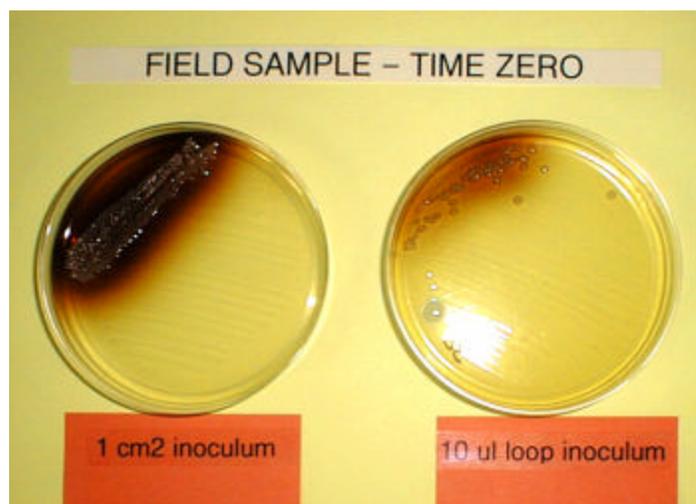
1. A larger kidney tissue, ~1 cm<sup>2</sup>, is used to inoculate BHIA plates. More tissue increased both the number of colonies and number of positive culture tests for *Aeromonas salmonicida*, especially in fish with low-level infections as determined by low colony forming units (CFU).

In this study, some positive fish would have been missed if 10ul loops were used to inoculate kidney tissues onto growth media.

Statistical analysis of the 15 pairs used presence or absence as the sensitivity measure rather than CFU's because of the difference in the amount of tissue sampled between treatments.

Wilcoxon's signed-ranks test did not detect a significance for *A. salmonicida*, or non-*A. salmonicida* isolates A, B, and C. For tests involving paired attribute data, at least six pairs with differences in the same direction would have been required for statistical significance. Only pairs from individuals 5 and 8 met this criteria. A larger sample size would be needed, or higher incidence of *A. salmonicida* in the population, to determine if presence or absence were statistically significant for this objective.

For reporting purposes, *A. salmonicida* would have been detected in the stock with either sampling method. However, apparent prevalence reported for the population would be lower than what was detected when larger kidney tissues were used during sampling. This could be very important for diagnostic cases where optimum sensitivity and rapid detection are important for effective disease control. It could also be important in accurately monitoring infections over the course of antibiotic treatment during an epizootic, or in broodstock populations over time.



**Figure 1. BHIA plate showing increased colony numbers obtained with 1 cm<sup>2</sup> kidney tissue, compared to 10ml inoculating loop from the same fish.**

**Objective #2 - Evaluate culture sensitivity of individual kidney tissues collected and inoculated at 0-HOUR (field inoculation) compared to 24-HOUR (laboratory inoculation) to determine if bacteria viability decreases over 24 hours.**

2. Kidney tissue is inoculated immediately onto BHIA, versus plating at 24-hours. Again, low level infections might be missed if samples are plated at 24 hours, and the reported prevalence for the population would be lower.

Statistical analysis of this paired design for *A.salmonicida* used CFUs as a sensitivity measure (same tissue was used for 0 and 24 hrs, in both the individual and pooled samples) Data were not normally distributed and the presence of many zero values prevented successful data transformation. Wilcoxon's signed-ranks test was applied and results were significant at the  $\alpha < .01$  (one tailed test). Samples processed at 0 hrs were positive more often than those sampled at 24 hrs.

For non-*A.salmonicida* isolates A, B, and C, only presence or absence (attributes) were available for measuring sensitivity. The Sign Test was applied to A, B, and C types as well as *A.salmonicida*. Results for type B (Non-fermenter) and *A.salmonicida* were significant at  $\alpha = .05$  (one-tailed) in favor of 0 hours. Types A and C did not exhibit significance.

**Objective #3 - Test culture sensitivity of individual kidney versus 5-pool kidney samples inoculated at either 0-HOUR (field) or 24-HOUR (laboratory) to determine if pooling tissues decreases culture sensitivity. (Also evaluate viral processing and use of "kidney pellet" as inoculum for BHIA plates).**

3. Bacteria is concentrated in 5-pool kidney samples by centrifugation (6000rpm for 10 mins) prior to inoculation onto BHIA plates. This concentration of bacteria in the pellet appears to be the significant factor in increased sensitivity (Elliott, 1997). Plating the tissues on growth media at 24-hours did not appear to be a factor in the increased sensitivity observed as data presented in Objective 2 contradicts this observation (1-pool kidney samples at 0-hour samples were more sensitive than samples inoculated at 24- hours).

This was also a paired design. Mean CFU's per fish were compared. The pooled means were not normally distributed, and contained too many zeros for transformation. It is interesting that the means obtained from the 1-P treatment were normal when the square-root transformation was applied. Wilcoxon's signed-ranks test was applied. Results were not significant ( $\alpha = .05$ ). They were very close to significant since there were five differences in the same direction (six is required). Again, larger sample size may be indicated to fully address this objective.

## References

Elliott, 1997. Comparison of Two Fluorescent Antibody Techniques (FATS) for Detection and Quantification of Rs in Coelomic Fluid. *Dis. Aq. Org.* 30; 37-43.

OIE Diagnostic Manual for Aquatic Animal Diseases. Third Edition, 2000.

Thoesen, John C. Editor. 1994. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. 4<sup>th</sup> Edition, Fish Health Section, American Fisheries Society.