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Comparison of individual and pooled sampling methods for detecting bacterial pathogens of fish

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Abstract. Examination of finfish populations for viral and bacterial pathogens is an important component of fish disease control programs worldwide. Two methods are commonly used for collecting tissue samples for bacteriological culture, the currently accepted standards for detection of bacterial fish pathogens. The method specified in the Office International des Epizooties Manual of Diagnostic Tests for Aquatic Animals permits combining renal and splenic tissues from as many as 5 fish into pooled samples. The American Fisheries Society (AFS) Blue Book/US Fish and Wildlife Service (USFWS) Inspection Manual specifies the use of a bacteriological loop for collecting samples from the kidney of individual fish. An alternative would be to more fully utilize the pooled samples taken for virology. If implemented, this approach would provide substantial savings in labor and materials. To compare the relative performance of the AFS/USFWS method and this alternative approach, cultures of *Yersinia ruckeri* were used to establish low-level infections in groups of rainbow trout (*Oncorhynchus mykiss*) that were sampled by both methods. *Yersinia ruckeri* was cultured from 22 of 37 groups by at least 1 method. The loop method yielded 18 positive groups, with 1 group positive in the loop samples but negative in the pooled samples. The pooled samples produced 21 positive groups, with 4 groups positive in the pooled samples but negative in the loop samples. There was statistically significant agreement (Spearman coefficient 0.80, $P < 0.001$) in the relative ability of the 2 sampling methods to permit detection of low-level bacterial infections of rainbow trout.

Key words: Bacteria; disease; fish; pooling; sampling; trout; *Yersinia.*

Introduction

Fish health inspections are performed to reduce the likelihood that a pathogen will be transferred from one location to another. The Office International des Epizooties (OIE) provides standard procedures for fish health inspections[5] that can be used by its member countries, whereas the American Fisheries Society (AFS) and US Fish and Wildlife Service (USFWS) have published similar procedures for use in the United States.[8] Both the OIE Manual and the AFS Bluebook/USFWS Inspection Manual provide these procedures for fish health inspections where the population size can be in the thousands or even millions of individual fish. To obtain a representative sample, large numbers of fish must often be evaluated. As a result, both manuals allow for pooling of samples for virological testing, up to a maximum of 5 fish per pool can be included to reduce the number of samples (but not numbers of fish) required for such inspections. Pooling for pathogen detection is commonly accepted for certain applications when testing humans and animals.[2,3,6,7] The OIE manual supports pooling of tissues for detection of bacterial fish pathogens. In contrast to its international counterpart, the current AFS Bluebook/USFWS Inspection Manual does not allow for pooling of samples for bacteriological testing.

A more efficient method of obtaining bacterial cultures for fish health inspections in the United States would be to more fully utilize the pooled samples taken for virological testing. This method differs from the OIE method in that the OIE requires 2 different sets of tissues separated at the time of collection: 1 for bacteriological testing and 1 for virological testing. These samples can be pools of up to 5 fish. The alternative method tested in this study would allow the sampler to take 1 set of tissues in the field. When tissue samples are taken for virology, a portion of kidney and spleen are aseptically collected and placed in a sterile bag or tube, and samples from up to 5 fish can be combined. These pooled samples are diluted, macerated, and centrifuged, and the supernatant is removed for virological testing. Typically, the pellet is discarded. However, a sterile inoculating loop can be used to retrieve a sample from the pellet for bacterial culture on the appropriate media.

This alternative method differs from the OIE method in that the pooled samples are not divided into those used for bacteriological and virological samples at the time the samples are collected from the fish. Presum-

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ably, the OIE specifies separating the samples to accommodate the option of placing samples taken for virology into transport media containing antibiotics. This step is taken if bacterial overgrowth is expected, which could cause problems in a tissue culture assay. However, bacterial overgrowth can be avoided by keeping the samples on ice (but not frozen) and processing the samples within 24–48 hours. If bacterial overgrowth cannot be prevented without the use of antibiotics, then a different sampling technique would be required.

Because most fish health laboratories in the United States pool samples for virology but do not pool bacteriological samples, the use of this alternative method for bacteriological testing could result in substantial savings in labor for sample collection and processing, as well as in the cost of supplies and culture media. Although pooling samples may be less optimal for determining the prevalence of a pathogen, it may be a viable option for certification inspections because the presence of a pathogen, not its prevalence, determines the status of a population. Accordingly, the authors conducted a series of experimental trials to determine whether a sample from the pellet of pooled renal/splenic tissue samples yielded the same assessment of bacterial infection status as individual kidney samples taken with an inoculating loop.

Materials and methods

A scenario was designed to compare bacteriological sampling method specified in the AFS Bluebook/USFWS Inspection Manual to the alternative method under conditions assumed to be the least favorable to the alternative method (Fig. 1). If a single fish with a low-level infection were to be combined with 4 uninfected fish, would the pooled sample test positive? The authors defined a low-level infection as one that would yield 20 colonies or fewer on the bacterial culture plate after an infected fish was sampled by 1 of the methods. Several preliminary trials were conducted to develop exposure levels and postexposure sample collection times to produce a population of fish with a low-level bacterial infection. To evaluate possible dilutional effects that may occur with pooled kidney/spleen samples, the tissues collected from an exposed fish were combined with those from 4 fish that were not exposed to the pathogen. In this manner, the authors attempted to reflect the potential loss of test sensitivity because of dilution of bacteria in a pooled sample containing a single positive fish. In addition, the authors attempted to address the possible interference in the growth of bacteria on culture from the inclusion of splenic tissues in the pooled sample.

All fish were tested by both methods. Because all nonexposed fish tested negative when samples were obtained with an inoculation loop from kidney (individual samples), only the culture results obtained from exposed individual fish were compared with results from the pooled sample containing that fish.

Experimental fish. Two-hundred rainbow trout (*Oncorhynchus mykiss*) were obtained from the Nisqually Trout Farm, Thurston County, Washington, and transported to the Western Fisheries Research Center in Seattle, Washington. The fish were housed in a 369-liter circular stock tank and maintained in pathogen-free freshwater at 16 C at a density index (DI) of 0.22, based on the formula DI = total weight of fish in kg ÷ volume of water in meters^3 ÷ average length of fish in millimeter. Before the experiment, a subsample of the fish was examined for external parasites (skin scrapes and gill clips) and bacterial pathogens (kidney cultures). The remaining fish were treated for external parasites with formalin at 200 ppm for 1 hr. Subsequently, skin and gill samples were examined, which failed to detect any external parasites. No bacterial pathogens were detected. The remaining fish were allowed to acclimate in the tank for 3 mo before the experiment. At the time of the study, 10 fish were collected randomly for length and weight measurements.

Preparation of innoculum. An isolate of *Yersinia ruckeri* was obtained from Clear Springs Food Inc., Buhl, ID. This isolate was selected because it had been used for vaccine development and a standard challenge protocol had been established. The isolate was cultured on multiple plates of Brain heart infusion agar (BHIA).[a] The bacterium was confirmed to be *Y. ruckeri* by a fluorescent antibody test (FAT).[b] During the log-growth phase, the bacteria were harvested by covering the plate with 10 ml of sterile saline, gentle agitation, and pipetting the resulting suspension into a 50-ml centrifuge tube. Five 10-fold serial dilutions made from 10^{-1} to 10^{-5} were prepared, and each concentration was injected intracelomically into a different fish. Two days later, the fish were euthanized with an overdose of tricaine methanesulfonate[c] (greater than 250 ppm). The fish were placed in right lateral recumbency and sprayed with 70% ethanol. The alcohol was in contact with the fish for 1–3 min before opening the body cavity with sterile instruments. Using a sterile inoculating loop, individual samples of the kidney and spleen were cultured on BHIA. The plates were streaked for isolation, and bacteria were cultured from 2 of the 5 fish. Of the colonies obtained, an isolate from the fish injected with the lowest concentration of organisms was selected for further growth in the challenge experiment to ensure that a virulent strain of *Y. ruckeri* was used.

Preparation of bacterial suspension. Five cultures of the freshly passed isolate of *Y. ruckeri* were prepared on plates of BHIA. During the log-growth phase, the bacteria were harvested by flooding the surface of the plate with 10–15 ml of sterile physiological saline (0.85%), gentle agitation, and pipetting the resulting suspension into a 50-ml centrifuge tube. Aliquots of the bacterial suspension were added to a 1-liter bottle containing sterile saline until the resulting suspension had a transmittance of 50% (T50%) at 640 nm when read using a colorimeter.[d] Four 100-fold serial dilutions were plated on BHIA to retrospectively estimate numbers of bacteria per milliliter of the suspension.

Challenge procedure. A 104.5 ml volume of the T50% solution was added to 731.5 ml of sterile saline. This solution was added to 46 liters of water in a 75-liter tank. The temperature of the water was 15 C, and the water was aerated. Forty rainbow trout were added to the challenge tank,

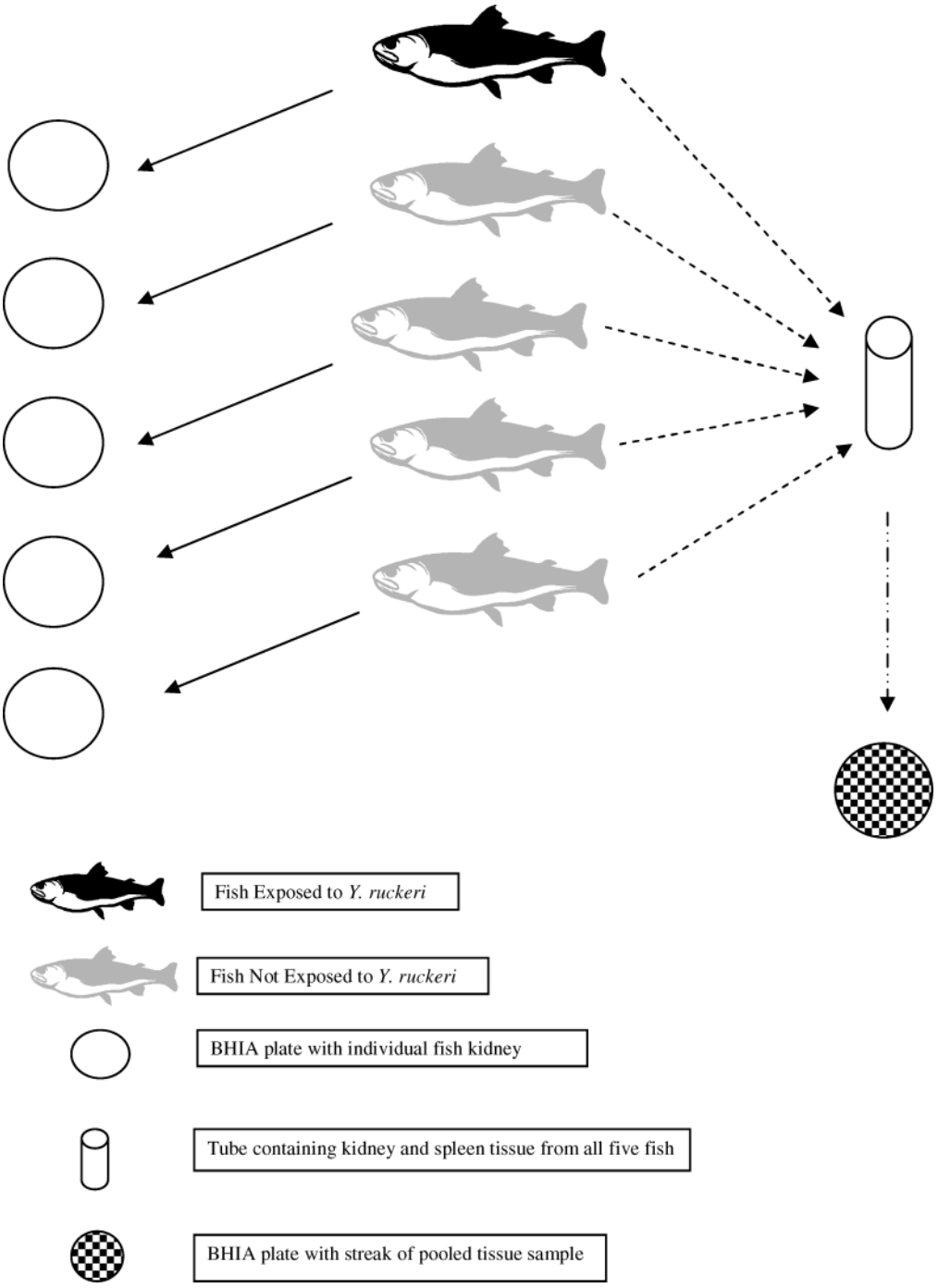

Figure 1. Overview of experimental design.

held for 1 hr, then divided into four 75-liter tanks provided with aeration and flowing, pathogen-free freshwater at 15 C. For the fish exposed to the bath treatment, on the basis of the above formula, the DI was 0.06. The nonexposed fish remained in the stock tank, but with 40 fewer fish remaining, the DI dropped to 0.18.

Sampling of fish. All fish were euthanized approximately 48 hrs after exposure of the exposed group using an overdose of tricaine methanesulfonate. Exposed and unexposed fish were not commingled before death. The fish were placed in right lateral recumbency and sprayed with 70% ethanol.[e] After 1–3 min, a midline incision was made using sterile scissors, and the spleen was removed using sterile forceps and placed in a separate sterile tube for use, later in the pooled assay. Sterile instruments were used on each fish to reduce the risk of cross contamination. The rack of tubes was held on ice for the duration of the sampling. The caudal end of the swim bladder was pulled away from the body wall using the forceps, and a sterile loop was inserted into the kidney approximately at the midpoint of the anterior and

posterior kidney. The sample was then plated on BHIA. Every fish was tested by the individual kidney loop method.

For the pooling method, each 5-fish sample consisted of relatively equal volumes of kidney and spleen tissues from 1 exposed fish combined with similar amounts of kidney and spleen tissues from 4 unexposed fish taken from the holding/stock tank. Using sterile technique, an approximately 3 mm^3 piece of kidney was removed from each exposed fish that had been sampled earlier as an individual fish by the loop method. The renal tissue was added to the tube containing splenic tissue from the same fish. The process was repeated using renal and splenic tissues from the negative fish until tissues from 5 fish (1 exposed and 4 negative) were included in each pool. On completion of sampling, the tubes were kept in a cooler on ice for transportation to the Olympia Fish Health Center (approximately 90 min drive), then transferred to a refrigerator and kept at 4 C overnight.

Processing of pooled samples. The day after collection, samples were diluted with 3 ml of Hanks balanced salt solution,[f] and the tissues were transferred into a sterile bag and homogenized using a laboratory blender.[g] A 1.5-ml aliquot of the mixture was transferred to a sterile 5-ml tube and centrifuged at 2,000 RCF for 20 min. The supernatants were discarded, and a sterile loop was used to obtain a sample from each pellet. After streaking the sample onto BHIA plates, the plates were incubated at room temperature for 96 hrs to detect the presence of *Y. ruckeri.* The identity of the bacteria from each sample was confirmed as *Y. ruckeri* by FAT. One colony from each plate was tested.

Statistical methods. Three groups were omitted from the analysis. In the first group, the exposed fish died prior to sampling and thus could not be used to create a pooled sample. The fish died overnight, and was too autolyzed to discern a cause of death. In the second group, the sampler inadvertently did not include the kidney from the infected fish in the pool, and in the third case, the final group only had 3 fish in the pool because of insufficient numbers of unexposed fish remaining in the stock tank. No mortalities occurred in the stock tank during the experiment.

Spearman rank correlation coefficient, corrected for ties, was used to determine the strength of association between the individual loop and 5-fish pool techniques. To calculate this correlation coefficient, the results from the 2 testing methods were ranked independently, on the basis of the number of colonies cultured in each group (i.e., the sample with the most colonies for that method had the highest rank). Because there were cases in which the number of colonies counted was equal within a method, a correction for ties was required. Cohen's kappa was also used to measure the agreement between the 2 methods in classifying samples as *Y. ruckeri* detected ($\geq$1 colony counted) versus not detected (zero colonies counted). A kappa value of zero indicates no agreement beyond that expected by chance, and kappa = 1.0 indicates perfect agreement.[1]

Results

The 10 fish that were randomly selected at the start of the experiment ranged in weight from 40.9 to 114.5 g ($\bar{x} \pm$ SD = 76.8 $\pm$ 18.8 g). The fork length ranged from 151 to 207 mm ($\bar{x} \pm$ SD = 186.5 $\pm$ 14.4 mm).

Using the average of the Karber and Reed-Muench methods, the concentration of the challenge bath was 4.6×10^6 bacteria/ml. This dose was sufficient to initiate infections that were detected by 1 or both methods in slightly over half of the exposed fish sampled at 48 hours after challenge.

Yersinia ruckeri was detected in a total of 22 of 37 groups by the loop or the pooling method (or both). Not all colonies on each plate were confirmed by FAT, but the colonies appeared uniform in color, texture, and growth characteristics. The loop method produced positive cultures from 18 groups; there was 1 group where the loop was positive and the corresponding pool was negative. The 5-fish pool method produced 21 positive groups; there were four 5-fish groups where the pooled sample was positive and the individual loop sample was negative (See Table 1).

There was a strong association between the results obtained with the loop and the 5-fish pool method (Spearman coefficient 0.80, $P < 0.001$). Correlation coefficients greater than 0.5 are considered to be large.[1] The value for Cohen's kappa was 0.73 ($P < 0.001$). Values greater than 0.7 are considered to indicate substantial agreement.[4] These analyses show that the 2 techniques produced similar ordering of samples from those with the most to those with the fewest colonies, and there was statistically significant agreement between the 2 techniques in their ability to allow detection of *Y. ruckeri.*

Discussion

This experiment was designed to compare the bacteriological sampling method specified in AFS Blue Book/USFWS Inspection Manual and a variation of the OIE sampling method under conditions assumed to be the least favorable to the novel method. The pooling method described in this study would reduce material and labor costs during collection and processing of samples for fish health inspections. Although it would have been ideal to test all 3 methods simultaneously, the amount of splenic tissue available per fish eliminated this option. Because this experiment was designed to determine whether this alternative method was a viable option in the United States, the authors focused on comparison with the AFS method.

Yersinia ruckeri was selected as a test organism because it is a gram-negative bacterium, a pathogen of regulatory significance, and has an established waterborne challenge protocol for research purposes. In this experiment, although the authors were not able to determine whether a given fish was truly negative (even if samples using both methods were negative), they

Table 1. Number of bacterial colonies obtained from groups of rainbow trout exposed to *Yersinia ruckeri* and sampled by 2 methods. Colony counts for each group reflect those of the 1 exposed individual fish in each pool (individual loop) and for the pool itself (5-fish pool) in which the tissues from the exposed fish were added to tissues from 4 unexposed fish.

Group	Individual loop Colonies*	5-Fish pools Colonies*
1	0	2
2	10	8
3	1	0
4	0	0
5	50	10
6	1	8
7	0	0
8	0	0
9	40	20
10	0	0
11	6	8
12	0	0
13	0	0
14	0	0
15	16	10
16	3	1
17	4	10
18	1	3
19	1	8
20	0	0
21	2	1
22	4	18
23	0	15
24	0	0
25	0	0
26	0	2
27	20	10
28	0	0
29	0	0
30	4	3
31	3	2
32	0	1
33	0	0
34	0	0
35	0	0
36	5	4
37	16	20

* Colony counts above 20 are estimates, otherwise exact counts are given.

were able to essentially rule out false positives by reducing the risk of cross contamination through the use of sterile instruments and by confirming the identity of *Y. ruckeri* with a FAT specific for *Y. ruckeri.*

Two primary concerns have slowed the adoption in the United States of pooling tissues for assessing the presence of bacterial pathogens in fish populations 1) the possible dilutional effect from combining 1 fish having a low-level infection with several negative fish which may result in pathogen levels that are below the detection limit of the assay and 2) the inactivation of a pathogen present in 1 fish by inhibitors introduced from other tissues (e.g., spleen) added to the same sample. This study was designed to address the first concern by combining tissues from a fish with a low-level of infection with tissues from 4 unexposed fish. It addressed the second concern by the inclusion of the splenic tissue in the pooled sample, with no net loss in ability to detect bacteria when compared with the loop method, which included only renal tissue.

In this study, detection of *Y. ruckeri* relied on bacterial culture for both individual and pooled samples. However, the 2 methods differed in the volume and type of tissues incorporated in the sample. Kidney/spleen samples were used in the pooled assay because they are the tissues routinely collected for virological testing. When using an inoculating loop directly in the kidney, particularly of small fish, it is extremely difficult to fill the loop with tissue because of the size and texture of the kidney. In contrast, once the tissues are diluted and homogenized, the inoculating loop is easily filled.

Typically, when culturing for bacteria, the greater the sample volume, the greater the number of colonies. A simple comparison of the number of colonies isolated using the 2 methods might be expected to favor the pooled method. To eliminate this bias, a rank comparison was performed. Another reason a direct comparison of colony counts would not be appropriate is the potential for focal infections in the fish kidney. A given sample taken by individual loop may range from highly positive to negative, depending on sampling location, whereas the homogenized sample using in the pooling method may yield a lower but more consistent colony count. Despite these differences, the number of groups yielding a positive bacterial culture showed strong agreement between the 2 sampling methods. In 4 of the 5 cases in which the 2 methods did not agree, the pooled sample yielded a positive detection, suggesting a lack of negative effects from dilution or from interference by inclusion of splenic tissues.

The globalization of economies and the speed with which live fish can be successfully transported has heightened the need for thorough fish health inspections and the standardization and validation of fish health assays. For international movement of aquatic animals, the OIE Aquatic Animal Health Code and the methods specified in the OIE Manual of Diagnostic Tests for Aquatic Animals are the recommended norms for adoption by member countries to help reduce the spread of certain aquatic animal diseases. Within these documents are sections on validation of diagnostic assays that address performance characteristics such as sensitivity, specificity, reproducibility, and reliability.

Because the actual infection status of the samples used in this study could not be known with certainty and because this study involved only the sampling

strategy used for the bacterial culture method for detection of fish pathogens, several of these standard performance measures could not be calculated accurately. However, under ''worst case'' conditions, the results from the pooling method outlined in this study showed a statistically significant agreement with results with the individual loop method. In fact, the 5-fish pool sampling method detected more positives than the individual loop method specified by the AFS Bluebook/USFWS Fish Health Inspection Manual. These findings indicate the AFS Bluebook/USFWS Fish Health Inspection Manual could be modified to allow the use of 5-fish pools for bacterial sampling, which would lead to substantial savings in time and materials.

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b. USFWS Fisheries Academy, Leetown, WV.
c. Western Chemical Inc., Ferndale, WA.
d. Bausch and Lomb Spectronic 20, now Thermo Electric Corporation, Waltham, MA.
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