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Use of ELISA for Monitoring Bacterial Kidney Disease in Naturally Spawning Chinook Salmon

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Glenda O'Connor
La Grande Fish Health Laboratory
Oregon Department of Fish and Wildlife
219 Badgley Hall
Eastern Oregon University
La Grande, Oregon 97850

and

Timothy L. Hoffnagle
Northeast Oregon Fish Research
Oregon Department of Fish and Wildlife
203 Badgley Hall
Eastern Oregon University
La Grande, Oregon 97850

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Abstract

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum* (Rs), is a serious problem among Pacific Northwest salmon hatcheries, including the concern that salmon reared in hatcheries may spread BKD to natural populations. In order to monitor the potential spread of this disease to salmon spawning in nature, a method must be available to collect and analyze tissues from naturally spawning salmon. Kidney tissue analyzed by enzyme-linked immunosorbent assay (ELISA) is the standard method to detect the presence of Rs in salmon sampled in hatcheries. We tested the ability to use ELISA on kidney tissue collected from intact carcasses recovered on the spawning grounds to monitor BKD in naturally spawning populations. We compared ELISA optical density (OD) values from kidney tissue that was subjected to simulated conditions to which kidney tissue would be exposed in a decomposing carcass and following collection during a spawning ground survey versus samples collected from freshly killed salmon at a hatchery. Mean ELISA OD levels were 1.060 for the samples prepared by the normal preparation and 1.115 for samples prepared by simulating spawning ground survey collection. We found no significant difference in mean ELISA optical density between the sample preparations and the relationship between them was nearly 1:1 (slope = 0.946). This demonstrates that BKD prevalence in natural populations can be monitored using ELISA conducted on samples from intact carcasses on spawning ground surveys. This will be an important tool for monitoring the effect of hatchery supplementation on naturally spawning salmon populations.

Introduction

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum* (Rs), is a widespread and serious concern among Pacific Northwest salmon hatcheries (Fryer and Lannan 1993; Maule et al. 1996). This Gram positive organism is very difficult to treat with antibiotics and can persist through the entire lifetime of a fish. Once established in a population, the organism can be transmitted from fish to fish (horizontal transmission; McKibben and Pascho 1999) and adult females that are infected with Rs can transmit the organism to their progeny (vertical transmission; Evelyn et al. 1986). Enzyme-linked immunosorbent assay (ELISA) is the standard serodiagnostic method used to detect the presence of Rs in salmon (Pascho et al. 1991). The ELISA detects the presence and amount of the Rs p57 antigen, or antigen F, a 57 kDa protein and the chief soluble antigen produced by Rs (Wiens and Kaattari 1989). It is found both on the surface of the bacterial cell and in a soluble extracellular form and plays a role in the virulence of this pathogen (Getchell et al. 1985; Turaga et al. 1987; Bruno 1988). The BKD ELISA is used at many hatcheries to screen adult salmon for BKD, allowing culling of eggs from Rs-infected females or segregation of their offspring, strategies which have proven effective in reducing BKD in hatchery populations by reducing vertical transmission (Pascho et al. 1991).

Hatcheries play an important part in the restoration and supplementation of threatened and endangered Pacific salmon stocks (Brannon et al. 2004). However, there are also concerns about hatcheries, including the potential to spread disease to natural populations (Hastein and Lindstad 1991; Waples 1999). Chinook salmon *Oncorhynchus tshawytscha* in the Grande Ronde and Imnaha basins of northeast Oregon are listed as threatened and hatchery programs were developed as early as 1982 to restore and supplement their populations (Carmichael and Messmer 1995). In the Grande Ronde Basin, the populations declined to levels so low that a captive broodstock program was developed to prevent imminent extirpation (Hoffnagle et al. 2003). In the Grande Ronde Basin Spring Chinook Salmon Captive Broodstock Program, BKD is the largest source of mortality, causing up to 63% of the total loss during rearing. At the time of spawning, as many as three-quarters of the females have indications of past or present Rs infection, as indicated by ELISA optical density (OD) levels > 0.4.

Because Rs can be vertically transmitted (Evelyn et al. 1986), when BKD is prevalent in a hatchery broodstock, managers must decide whether to cull the eggs from positive females or to rear and release their offspring in the anticipation that at least some will survive and help restore the threatened populations. However, releasing offspring from females with a high ELISA OD and a high risk of Rs infection raises the concern of increasing the prevalence of Rs in natural populations. In the Captive Broodstock Program, the need to conserve as much of the genetic diversity as possible has sometimes resulted in the release of offspring from females with high ELISA OD levels, despite the possibility that the females may have transmitted the disease to at least some of their offspring and more juveniles may have been subsequently infected horizontally. Since all returning adults that were produced by the Captive Broodstock Program are allowed to spawn in nature, a method is needed to monitor the prevalence and incidence of BKD in naturally spawning adults and determine whether these hatchery origin salmon are spreading the disease to natural salmon. This type of monitoring program is equally important for any hatchery supplementation program where BKD is a concern (Waples 1999).

Beginning in 2000, kidney samples from pre-and post-spawning mortalities in the John Day River Basin were collected to test for Rs infection by ELISA. Sampling of dead, naturally

spawning Chinook salmon has since expanded to include streams in the Grande Ronde Basin to monitor the effect, if any, of the release of Captive Broodstock offspring into nature. Salmon carcasses found in streams are often degraded at the time of collection and individuals collecting the tissue samples are not trained lab technicians, nor are stream-side collections an ideal environment for sterile tissue collections. Consideration of these factors has led to speculation as to the validity of ELISA results obtained under such circumstances. When analyzing kidney samples from spawning ground survey carcasses, we must be concerned that the length of time since the death of the fish and the manner in which the samples are handled prior to arrival at the lab may affect ELISA results.

The purpose of this study was to determine whether ELISA is a valid method for monitoring BKD prevalence in naturally spawning salmon and whether these results are comparable to those collected from freshly killed salmon. Specifically, we compared ELISA OD levels between a tissue sample prepared by the standard method for hatchery monitoring (normal) versus a sample, from the same fish, subjected to an approximation of field conditions: a carcass laying in the stream for several days following death and then the sample being carried in a backpack after being collected (spawning ground survey). In this way, we could determine whether ELISA can be used on kidney samples collected from intact carcasses recovered on the spawning grounds to measure the infection level of salmon in nature and as a means by which we can monitor BKD in nature and compare with salmon spawned at hatcheries.

Methods

We collected kidney tissue samples from 25 mature Chinook salmon males during spawning of the Grande Ronde Basin Spring Chinook Salmon Captive Broodstock Program in September 2005 at Bonneville Fish Hatchery. We used these fish because of the high likelihood of obtaining samples with a wide range of ELISA OD levels. We collected duplicate adjacent kidney samples from each salmon and placed each sample in separate Whirl-Pak bags. We froze (-5° C) the samples for 24-48 hours until we transported them on ice, to the Oregon Department of Fish and Wildlife La Grande Fish Health Laboratory, where we stored them at -29° C. Tissue sample size ranged from 0.56 – 2.57 g.

On 11-13 October 2005, we exposed one of the duplicate samples to the likely temperature regime to which a kidney sample would be subjected when decomposing in a northeast Oregon stream and collected during a spawning ground survey. We placed this sample in a 16° C incubator for 48 hours to simulate the maximum temperature to which a salmon carcass in northeast Oregon is likely to be exposed prior to recovery (1 – 4 days in a stream with diel temperature fluctuating between 6-20° C). We then removed the samples from the incubator and placed them in a drying oven at 27.8° C for 6 hours to simulate the time that a sample might spend in a backpack while the survey was being conducted. Following the spawning ground survey simulation, we stored the samples at -29° C for storage until they could be processed and analyzed by ELISA, as they would be handled following spawning ground survey collection.

On 25 October 2005, we processed all samples using the standard protocol for kidney ELISA preparations – samples collected for standard hatchery monitoring would remain frozen until processing and analysis. We added PBS-Tween 20 to each sample bag to create a 1:3 tissue to diluent dilution, then crushed this mixture under a marble rolling pin, and placed it in a Seward Stomacher 80 for one minute to disrupt tissue and facilitate antigen release to the

supernatant. We then poured the sample, 1.8 mL +/-0.2mL, into a labeled 2 mL screw cap vial, incubated it in a 100° C water bath for 10 minutes, cooled it to room temperature, centrifuged it for four minutes at 10x G and returned it to the -28.8° C freezer. We ran the ELISA on 28 October 2005 for seven of the 25 samples and on 9 November for the remaining 18 samples. We ran two replicate samples for each tissue sample and we assayed the paired (spawning ground survey simulation and normal) samples on the same day in adjacent wells and included a buffer control (PBS-Tween 20) and a 1:3 dilution negative kidney tissue control for both runs. Mean replicate values of 0.056 and 0.054 for the buffer control and 0.062 and 0.062 for the negative tissue control were within normal ranges and variation between replicates within samples was also low. Mean OD difference between sample replicates was 0.030 (2.7% of the mean ELISA OD value) and the mean variance was 0.0015.

We compared differences in mean ELISA OD levels between the normal and spawning ground survey preparations by t-test and we examined the relationship between the two sample preparations by regression (Sokal and Rohlf 1996). Additionally, we used a t-test to determine whether the slope of the relationship between the normal and spawning ground survey preparations differed from 1 (Neter et al. 1990). We considered differences between means to be significant at $\alpha < 0.05$.

Results

Mean ELISA OD was 1.060 for the samples prepared by the normal preparation method and 1.115 for samples prepared by simulating spawning ground survey collection (Table 1). We found no difference in mean ELISA OD between the two sample preparations ($P = 0.8311$) and their relationship highly significant ($P < 0.0001$; $r^2 = 0.925$; slope = 0.946) (Figure 1). If two outlying samples were removed (samples CC 24 and CC 51), then the relationship improved further ($P < 0.0001$; $r^2 = 0.986$; slope = 0.969). Although the calculated slopes of the lines reflecting the relationship between the normal and spawning ground survey preparations did not equal 1, this relationship did not significantly differ from a 1:1 relationship (P=0.3509).

Discussion

Our results demonstrate that kidney tissue can be collected from intact carcasses on spawning ground surveys and analyzed by ELISA and that these data can be reliably used to monitor BKD prevalence in natural populations. Also, kidney tissue samples properly collected under field conditions can be directly compared with samples collected from freshly killed salmon.

We found no evidence of a degradation of the p57 antigen during two days of simulating carcass deterioration and subsequent sample collection or a loss in the ability of ELISA to detect the antigen after such a period. Previous research has also found the p57 antigen to remain stable at elevated tempertures. Getchell et al. (1985) demonstrated that the p57 antigen was heat stable for 30 min at 100° C. Pascho et al. (1997) reported that the p57 antigen was still detectable by ELISA in hematopoietic tissues of rainbow trout that received an intraperitoneal bacterin injection and were held at 12° C for more than three months. Conversely, Griffiths and Lynch (1991) determined that p57 found in extracellular products is autolytic with regard to time and

Table 1. ELISA OD levels and the difference between them for 25 kidney samples prepared by the normal preparation and those prepared by simulating collection from carcasses on spawning ground surveys (SGS).

Fish number	Spawn date	ELISA OD		Difference (Normal - SGS)
		Normal	SGS	
CC 05	7 SEP 2005	0.088	0.085	0.003
CC 10	7 SEP 2005	1.501	1.546	0.045
CC 11	7 SEP 2005	1.856	2.237	0.381
GR 08	14 SEP 2005	0.137	0.189	0.052
GR 24	14 SEP 2005	1.911	1.906	0.005
LR 02	14 SEP 2005	1.699	1.535	0.164
CC 19	14 SEP 2005	0.073	0.105	0.032
CC 24	14 SEP 2005	1.943	1.219	0.725
CC 23	14 SEP 2005	0.260	0.499	0.239
CC 25	14 SEP 2005	2.046	2.000	0.046
CC 30	14 SEP 2005	1.930	1.921	0.009
CC 32	20 SEP 2005	0.083	0.103	0.020
CC 35	20 SEP 2005	0.189	0.342	0.154
CC 39	20 SEP 2005	2.003	2.143	0.140
CC 41	20 SEP 2005	2.017	2.159	0.142
CC 48	20 SEP 2005	1.583	1.508	0.075
CC 51	20 SEP 2005	0.955	1.800	0.845
CC 65	20 SEP 2005	0.146	0.179	0.033
CC 71	20 SEP 2005	0.078	0.073	0.005
CC 76	20 SEP 2005	2.369	2.525	0.157
CC 78	20 SEP 2005	2.184	2.293	0.109
CC 79	20 SEP 2005	1.216	1.266	0.051
LR 31	27 SEP 2005	0.076	0.072	0.004
LR 36	27 SEP 2005	0.098	0.091	0.007
LR 64	27 SEP 2005	0.067	0.068	0.001
Mean		1.0603	1.1146	0.0543
Standard deviation		0.8982	0.9127	0.2485
Minimum		0.067	0.068	0.001
Maximum		2.369	2.525	0.845

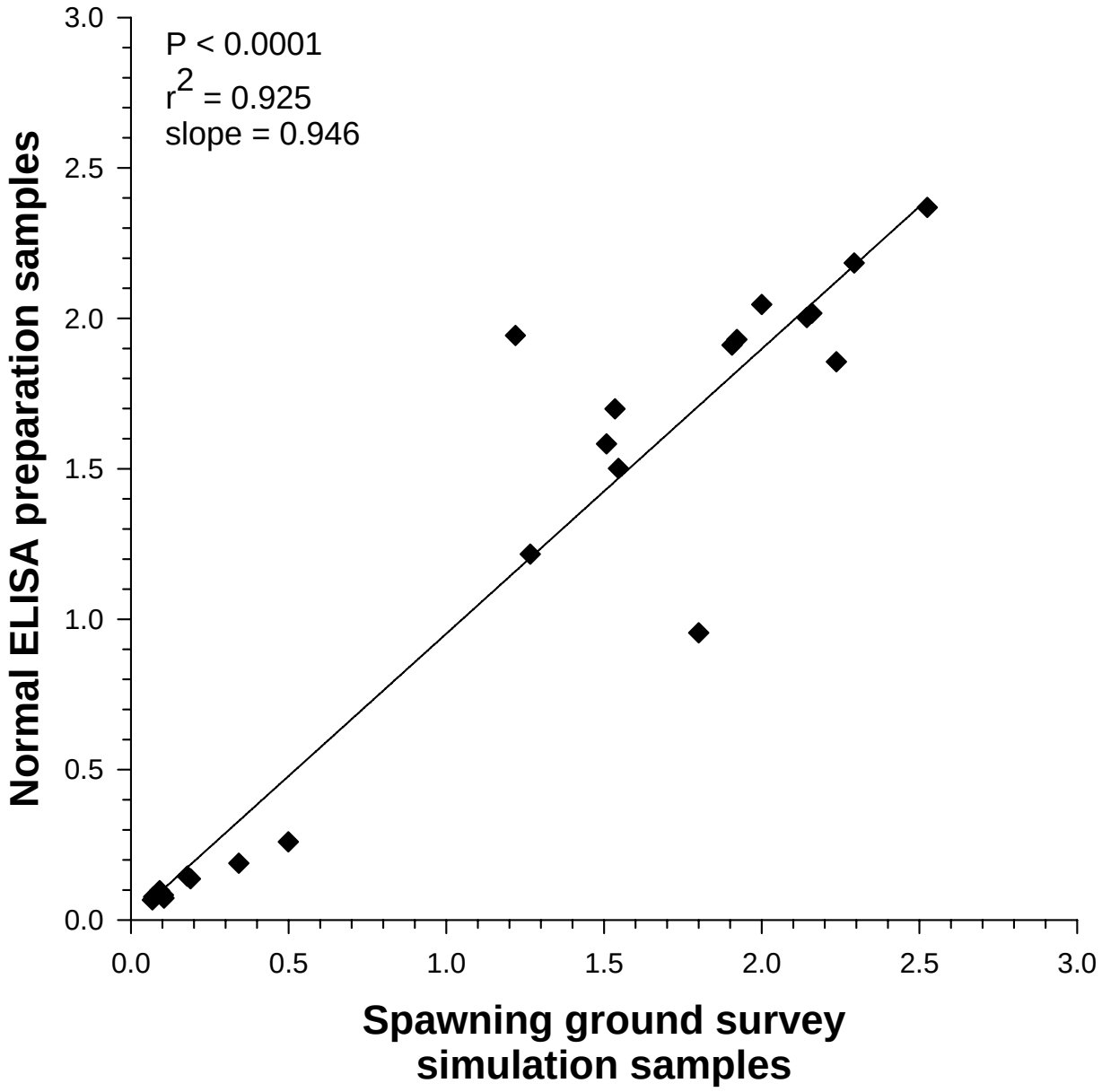

Figure 1. Relationship between ELISA OD for 25 kidney samples prepared by the normal preparation and those prepared by simulating collection from carcasses on spawning ground surveys.

increased temperature, breaking down into several distinct degradation products over a 10-h incubation period at temperatures of 25°C and 30°C. It is possible that the partial degradation of the simulation temperatures increased the availability of epitopes for recognition by the polyclonal antibody used in the ELISA (L. Rhodes, NOAA Fisheries, personal communication) because the spawning ground survey simulation samples generally had a higher ELISA OD

value (mean = 5.4%) than the normal preparations. However, the difference in mean ELISA OD value was not statistically significant and we tested the regression slope (0.946) and found that it did not differ from a slope of 1. Therefore, we feel that a 5.4% difference in ELISA OD level, even if it is real and not a statistical anomaly, will not affect the use of ELISA for the purpose of monitoring BKD prevalence or comparing its prevalence between spawning samples collected during a ground survey vs. the more sterile conditions of a hatchery.

For two of 25 samples in our experiment, there was a large difference in ELISA OD values between the normal preparation and spawning ground simulation (one higher in the spawning ground survey and the other in the normal preparation). It is noteworthy that both samples (CC24 and CC51) were in the clinical ELISA range (≥ 1) (Figure 1). Fish in this category are considered as having BKD and it is possible that an early stage granulomatous lesion was forming (Wiens and Kaattari 1999). These granulomas appear as whitish-grey nodules which vary in size and may have concentrated antibody-antigen complexes associated with them (Bruno 1986). Additionally, the distribution of Rs cells and p57 antigen within an infected kidney may not always be homogeneous and this uneven distribution is more pronounced in mature salmonids due to their larger kidneys (Meyers el al. 1993). Therefore, it seems likely that, although they were taken proximally, one of these paired samples contained a lesion, which resulted in a higher concentration of the p57 antigen and a higher ELISA OD value.

The Oregon Department of Fish and Wildlife has been collecting kidney samples from Chinook salmon carcasses on spawning ground surveys in the John Day River since 2000 and in the Grande Ronde and Imnaha rivers since 2004. Although there are no broodstock collections from the John Day Basin, ELISA OD levels have been similar to those found during spawning of hatchery broodstock from other streams in northeast Oregon (Wilson et al. 2002a; b). In the Grande Ronde Basin, we found no difference in mean ELISA OD levels between samples from the same populations collected during spawning ground surveys versus those collected during broodstock spawning at Lookingglass Fish Hatchery (ODFW unpublished data). These monitoring efforts and the present study show that BKD prevalence in natural populations can be effectively monitored using ELISA conducted on samples collected from intact carcasses. Additionally, fishery and hatchery managers can compare BKD prevalence in hatchery-spawned salmon with those spawning naturally. This will be an important tool for monitoring the effect of hatchery supplementation on naturally spawning salmon populations.

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