Journal of Veterinary Diagnostic Investigation
25(3) 341–351

DOI: 10.1177/1040638713482124
jvdi.sagepub.com

Sensitivity and specificity of histology for diagnoses of four common pathogens and detection of nontarget pathogens in adult Chinook salmon (*Oncorhynchus tshawytscha*) in fresh water

Michael L. Kent,[1] Susan Benda, Sophie St-Hilaire, Carl B. Schreck

Abstract. Histology is often underutilized in aquatic animal disease screening and diagnostics. The agreement between histological classifications of infection and results using diagnostic testing from the American Fisheries Society's Blue Book was conducted with 4 common salmon pathogens: *Aeromonas salmonicida*, *Renibacterium salmoninarum*, *Ceratomyxa shasta*, and *Nanophyetus salmincola*. Adult Chinook salmon (*Oncorhynchus tshawytscha*) in Oregon were evaluated, and agreement between tests was calculated. Live and dead (both pre- and postspawning) salmon were collected from the Willamette River, Oregon, its tributaries, the Willamette Hatchery, and after holding in cool, pathogen-free water during maturation at Oregon State University. Sensitivity and specificity of histology compared to Blue Book methods for all fish, live fish only, and dead (pre- and postspawned combined) fish only were, respectively, as follows: *A. salmonicida* ($n = 105$): specificity 87.5%, 87.5%, 87.5% and sensitivity 38.6%, 14.8%, 60.0%; *R. salmoninarum* ($n = 111$): specificity 91.9%, 85.7%, 97.7% and sensitivity 16.0%, 7.1%, 27.2%; *C. shasta* ($n = 136$): specificity 56.0%, 63.3%, 28.6% and sensitivity 83.3%, 86.2%, 71.4%; *N. salmincola* ($n = 228$): specificity 68.2%, 66.7%, not possible to calculate for dead fish and sensitivity 83.5%, 80.5%, 87.3%. The specificity was good for bacterial pathogens. This was not the case for *C. shasta*, likely due to detection of presporogenic forms only by histology. Sensitivity of histology for bacterial pathogens was low with the exception of dead fish with *A. salmonicida*. Kappa analysis for agreement between Blue Book and histology methods was poor to moderate. However, histological observations revealed the presence of other pathogens that would not be detected by other methods.

Key words: *Aeromonas*; *Ceratomyxa*; Chinook salmon; *Nanophyetus*; *Renibacterium*.

Introduction

Histology is often used for disease investigation in terrestrial animals but is less frequently used with investigations that involve fishes, particularly salmonids in public hatcheries in the United States. This is because the pathogens of captive salmonids are well recognized, and morbidity events are usually investigated with wet mount preparations of tissues for parasites and a variety of microbiological diagnostic tests that target specific known pathogens.[1] Histology, however, has the advantage of identifying a wide variety of expected and unknown pathogens, as well as lesions. Therefore, this method provides a powerful tool for establishing the cause of morbidity at a tissue and organ level.

Given that other diagnostic tests, such as polymerase chain reaction (PCR) and culture, are generally more sensitive and specific than histology for detecting pathogens in tissues, the latter is less frequently used for surveys for specific pathogens in large populations of clinically normal animals. Nevertheless, a few studies have used histology as a primary method for screening populations of fish for a wide variety of pathogens and lesions[7,13,18,32,44,45] or when toxicopathic changes are a target endpoint.[30,48] Successful studies using histology to survey for pathogens and associated lesions in salmonids have been reported in the Pacific Northwest of the United States[22,28,44] and in British Columbia, Canada.[45] Histological changes were also an endpoint in a recent investigation of cause of mortality in coho salmon (*Oncorhynchus kisutch*),[46] and a previous study[8] used histology to describe lesions in sockeye salmon (*Oncorhynchus nerka*) associated with prespawn mortality (PSM). For the past decade, PSM of adult Chinook salmon in the Willamette

From the Departments of Microbiology (Kent) and Fisheries and Wildlife (Benda, Schreck), and the Oregon Cooperative Fish and Wildlife Research Unit, U.S. Geological Survey Department of Fisheries and Wildlife (Schreck), Oregon State University, Corvallis, OR; and Atlantic College of Veterinary Medicine, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada (St-Hilaire).

[1]Corresponding Author: Michael L. Kent, Department of Microbiology, Oregon State University, 220 Nash Hall, Corvallis, OR 97331. Michael.Kent@oregonstate.edu

River system, Oregon, has been extremely elevated (≤90% in some cases).[26] Therefore, an ongoing multiyear study investigating PSM using histology to compare lesions and pathogens in PSM fish to asymptomatic prespawn and postspawn salmon was implemented. The sampling provided an opportunity to compare histology with other diagnostic tests.

Diagnostic methods described in the American Fisheries Society Fish Health Section (AFS-FHS) Blue Book[1] (henceforth referred to as the Blue Book) represent a collection of recommended protocols used by most fish health laboratories in the United States for identification of the most serious pathogens found in salmonid and other food fishes. The aim of the present study was to compare the effectiveness of histology to Blue Book methods for detection of 4 common pathogens of salmon: *Aeromonas salmonicida*, *Renibacterium salmoninarum*, *Ceratomyxa shasta*, and *Nanophyetus salmincola*.

Material and methods

Samples and diagnostic methods

A range of clinical and subclinical Chinook salmon from the Willamette River system, Oregon, was used to compare histological findings to the traditional Blue Book protocols. These protocols are contained in 2 sections on diagnostics, Diagnostic Procedures for Finfish and Shellfish Pathogens and U.S. Fish and Wildlife Services/AFS-FHS Standard Procedures for the Aquatic Animal Health Inspections,[1,50] and are consistent with the presumptive and confirmatory diagnostic tests set forth by other countries and organizations, including the World Organization for Animal Health (OIE).[53]

Adult Chinook salmon were collected from the Willamette River, its tributaries, and the Willamette Hatchery on this watershed between May and September 2010 and 2011. Samples included dead fish (prespawn mortalities and, rarely, postspawn mortalities), healthy fish collected midsummer, and postspawn fish collected in September. Dead fish were collected from the rivers by staff from the Oregon Department of Fish and Wildlife (ODFW) or colleagues from Idaho State University, Moscow, Idaho. Healthy fish were collected from fish traps at either the Willamette Falls or Dexter fish facilities on the Willamette River, and postspawned fish were collected from either the river or the Willamette Hatchery. Also included were samples of adult Chinook that were collected from the river early in the summer and held at the Fish Performance and Genetics Laboratory (FPGL; Oregon State University, Corvallis, Oregon) until death before spawning or euthanasia after spawning. For the "dead fish," only fish that were deemed to be relatively fresh (i.e., recently died) by the presence of red color in the gill were included in the study. Following collection, fish from all locations were placed in individual plastic bags, on ice, and then transported to Oregon State University for necropsy, which was performed on all fish within 24 hr of collection.

Histology

Heart, brain, gills, liver, spleen, kidney, pyloric caeca, and lower intestine samples were preserved in 10% buffered formalin. Tissues were then processed using standard histological methods and stained with hematoxylin and eosin. Four slides were prepared from tissues of each fish to assure adequate amounts of tissue for analysis as follows: 1) posterior kidney, liver, spleen, heart ventricle; 2) cross-sections of several pyloric cecae and lower intestine; 3) 3–5 gill filaments including the base; and 4) brain. Special stains, such as periodic acid–Schiff and Gram stain, often allow for visualization of *R. salmoninarum* in tissue sections.[19,27] Previous observations by the authors have found that this bacterium is inconsistently positive with these stains in sections, and thus special stains were not employed in the present study.

Diagnostic criteria for traditional tests and histology

Traditional microbiological tests were performed in collaboration with staff at the Fish Health Services (ODFW; Corvallis, Oregon), using the laboratory's routine conducted Blue Book methods. Specific tests for each pathogen are described in the following.

Aeromonas salmonicida. For the traditional test, inocula from excretory kidneys were obtained and streaked on tryptic soy agar (TSA) plates incubated at 18°C for 5 to 7 days. A sample was considered positive when brown, diffuse pigment was associated with colonies that tested oxidase positive. Infections by *A. salmonicida* at the histological level presented with a distinct pattern of bacterial colonies compared to most other Gram-negative bacterial infections of fish. Small bacilli bacterial colonies can occur in several organs (spleen, liver, kidney, gill, heart, and intestine) and are characterized by a distinct lack of inflammatory response and minimal or no associated necrosis.[11,19,24]

Renibacterium salmoninarum. The Blue Book lists the direct fluorescent antibody test (DFAT), enzyme-linked immunosorbent assay (ELISA), PCR, and culture as confirmatory tests, while section 2 of the Blue Book[50] restricts confirmatory diagnosis to the DFAT. The ELISA p57 antigen test, using a polyclonal antibody, was used as the traditional test for *R. salmoninarum* following the protocol as described in the Blue Book[1] and was conducted by ODFW Fish Health Services laboratory as they have an ongoing project that entails ELISA testing on fish from the Willamette Hatchery, Oregon. The tests were conducted with frozen kidney samples from the samples collected from the

river system or the FPGL. As this is a quantitative test, 4 categories of antigen based on optical density were designated as follows: negative = <0.100; low = 0.100–0.199, medium = 0.200–0.499, high = >0.5000.

Several fish were diagnosed as ELISA positive and histology negative, or vice versa. Therefore, kidneys of 24 fish were examined by the DFAT[4] in an attempt to elucidate the cause of the discrepancy. Previously frozen kidney tissue was homogenized with a stomacher in plastic bags, and 10 µl of the resulting fluid was spread thinly on a slide. Slides were processed as described previously[4] and read with a fluorescent microscope by ODFW staff, with no prior knowledge of histology or ELISA results. Slides were scored based on numbers of fluorescing bacteria observed per field at 1,000× (Table 3). For the categorization of infections using the DFAT, all fish with any evidence of bacteria were considered positive when they had a score ≤1+. Subsequently, the kappa analysis was re-run using a different positive cutoff where only the fish that had a score >1+ were categorized as positive.

Bacterial kidney disease (BKD), caused by *R. salmoninarum*, is characterized at the histological level by multifocal, chronic, granulomatous inflammation, particularly in the kidney. Diagnosis of *R. salmoninarum* infection (i.e., BKD) was made when focal or locally extensive chronic inflammation or granulomas were observed in the kidney, liver, or spleen.[20,38] For histology therefore, tissues scored as positive were those with nonencapsulated granulomatous lesions in the kidney, spleen, or liver in the absence of parasites.

Ceratomyxa shasta. This pathogenic myxozoan targets the gastrointestinal tract, where it is associated with severe damage to the pyloric cecae, stomach, and lower intestine.[5,23] The lesions are associated with presporogenic forms and myxospores, and severe infections extend through all layers of the gut and progress to extraintestinal organs. Presumptive diagnosis of *C. shasta* in the Blue Book is based on detection of myxospores in wet mount preparations of the intestine.[5] The confirmatory diagnosis is obtained on observing myxospores of the appropriate size and morphology. Confirmatory diagnosis can also be obtained by a monoclonal antibody test or PCR tests, which are described in the Blue Book.[1,50]

For the present study, wet mount examinations of lower intestine for the presence of *C. shasta* were performed using standard procedures in concordance with the Blue Book,[5] and the preparation was examined for 2 min at 200× magnification before it was designated as negative. For histology, the parasite had to be identified in the intestinal epithelium as basophilic, spherical, multicellular organisms with small nuclei, typical of myxozoan presporogenic stages.[5,6,23]

Nanophyetus salmincola. The metacercarial stage of *N. salmincola* is a common parasite of salmonid fishes in western Oregon, Washington, and northern California.[22,34] The infection is contracted in freshwater areas where the intermediate snail host *Juga plicifera* is present. Metacercariae are found in essentially any organ and are particularly common in the kidney. Hence, wet mounts (tissue squash preparations) is the most common method used for screening salmon populations,[2,25] but metacercariae of *N. salmincola* are also easily identified in histological sections.[20,44] Enumeration of *N. salmincola* was conducted by examination of approximately 0.5–1 g of kidney tissues pressed between clear plastic plates as described previously[21] and examined using a compound microscope at 25× magnification with low light and a lowered condenser to enhance contrast. Metacercariae were identified as *N. salmincola* by the presence of a large, opaque subspherical body in the posterior region, corresponding to the excretory vesicle.[20,21]

Diagnostic test comparisons

Histology was compared to the Blue Book[1] diagnostic procedure because such procedures are the traditional tests used to screen subclinical populations. The tests are used by most governmental fish health laboratories in the United States, and section 2 of the Blue Book is comprised of the specific protocols for these tests that U.S. Fish and Wildlife laboratories are required to follow. Considering the wide acceptance of the Blue Book protocols, particularly by regulatory agencies, such protocols were designated as the gold standard in the present study. The kappa statistic (test for overall agreement) for histology and the Blue Book–approved test for each pathogen of interest were initially calculated separately using GraphPad QuickCalcs (http://graphpad.com/quickcalcs/Kappa2.cfm). Due to the generally poor agreement between tests (Table 2), the agreement for infected (sensitivity) and uninfected (specificity) fish between histology and the Blue Book–preferred test was assessed for each pathogen separately. This was performed by dividing the number of infected or uninfected fish found by histology by the total number of infected or uninfected fish as determined by the Blue Book–preferred test; the calculation researchers used to identify where the disagreement between tests was occurring. The 95% confidence intervals for sensitivity and specificity of histology based on the results of the standard tests described in the Blue Book were determined for each pathogen using the Clinical Calculator 1 (http://www.vassarstats.net/clin1.html). In addition to calculating the overall agreement for infected and uninfected fish when all samples were included in the analysis, these measures were also calculated for fish that were found dead (clinical population) and for healthy fish separately. The sensitivity and specificity of histology for clinical and subclinical populations were found by comparing the 95% confidence intervals.

In addition, for *R. salmoninarum*, because 2 tests are approved in the Blue Book, the agreement between histology and both of these tests (DFAT and ELISA) were calculated separately. Further, the overall agreement was calculated

Table 1. Histology and traditional test diagnostic results for live, dead, and total adult Chinook salmon (*Oncorhynchus tshawytscha*) in which fish were examined by both methods.*

	Aeromonas salmonicida			*Renibacterium salmoninarum*			*Ceratomyxa shasta*			*Nanophyetus salmincola*		
	Total	Live	Dead	Total	Live	Dead	Total	Live	Dead	Total	Live	Dead
Positive by both tests	22	4	18	4	1	3	30	25	5	172	103	69
Histology –, traditional +	35	23	12	21	13	8	6	4	2	34	25	10
Histology +, traditional –	6	2	4	7	6	1	44	29	15	7	7	0
Negative by both tests	42	14	28	79	36	3	56	50	6	15	14	1

* Traditional tests (Blue Book[1]) for *Aeromonas salmonicida*: culture on tryptic soy agar; *Renibacterium salmoninarum*: enzyme-linked immunosorbent assay; *Ceratomyxa shasta* and *Nanophyetus salmincola*: wet mount examinations.

using a kappa statistic between the 2 approved Blue Book tests.

Results

A total of 259 fish were used for the analyses. Of these fish, 153 were sampled shortly after euthanasia from the field, the Willamette Hatchery, or the FPGL (Table 1), and the remainder were samples collected from dead fish from either the field or the FPGL. The results from histology for the detection of pathogens had poor to moderate agreement with the Blue Book tests. In general, the bacterial agents had lower overall agreement than the parasitic pathogens, with the exception of *A. salmonicida* in the samples from dead fish (Table 2).

Aeromonas salmonicida

Histology. Fish diagnosed as positive for *A. salmonicida* infections exhibited bacterial colonies in the spleen, kidney, liver, heart, intestine, and gills (Fig. 1). The colonies were associated with minimal or no inflammation and appeared similar regardless if the fish were collected live (Fig. 1a) or dead (Fig. 1b). Tissues from dead fish occasionally exhibited a zone of postmortem autolysis associated with the bacterial colonies.

Comparison. Infections by this bacterium were diagnosed in several live and dead fish by culture, and less frequently in live fish by histology (Table 1). The overall kappa statistic showed poor agreement but was improved when only dead fish were evaluated (Table 2). The ability of histology to detect fish that tested positive by culture was relatively poor (Table 2), as 35 of 57 (61.4%) culture-positive fish were negative by histology. This was significantly improved (from 14.8% to 60%) when only dead fish were used for analysis (Table 2). Histology results were similar to culture results approximately 87% of the time when cultures were negative for *A. salmonicida*, regardless of the group designations (Table 2).

Table 2. Agreement between histology and the recommended Blue Book[1] diagnostic test: *Nanophyetus salmincola*, wet mounts of kidney; *Ceratomyxa shasta*, wet mounts of intestine; *Aeromonas salmonicida*, culture on tryptic soy agar with oxidase-positive colonies; and *Renibacterium salmoninarum*, enzyme-linked immunosorbent assay (ELISA) to p57 protein from kidney and direct fluorescent antibody test (DFAT; all fish only).*

	Kappa statistic	Sensitivity based on fish positive by Blue Book method (%)	Specificity based on fish negative by Blue Book method (%)
A. salmonicida			
All fish	0.249	38.6 (26.3, 52.4)	87.5 (74.1, 94.8)
Live	0.018	14.8 (4.9, 34.6)	87.5 (60.4, 97.8)
Dead	0.478	60.0 (40.8, 76.8)	87.5 (70.1, 95.9)
R. salmoninarum			
All fish ELISA	0.098	16.0 (5.2, 36.9)	91.9 (83.4, 96.4)
Live ELISA	0.085	7.1 (0.4, 35.8)	85.7(70.8, 94.1)
Dead ELIA	0.014	27.2 (7.3, 60.6)	97.7 (86.5, 99.9)
All fish DFAT	0.290	35.3 (15.3, 61.4)	71.1(30.3, 94.9)
C. shasta			
All fish	0.292	83.3 (66.5, 93.0)	56.0 (45.7, 65.8)
Live	0.388	86.2 (67.4, 95.5)	63.3 (51.6, 73.6)
Dead	0	71.4 (30.3, 94.9)	28.6 (12.2, 52.3)
N. salmincola			
All	0.333	83.5 (77.6, 88.2)	68.2 (45.1, 85.3)
Live	0.347	80.5 (72.3, 86.7)	66.7 (43.1, 84.5)
Dead	0.147	87.3 (77.5, 93.4)	Not possible to calculate

* Numbers in parentheses are confidence intervals. Results are reported for all fish; fish collected "live," in which tissues were collected shortly after euthanasia; and as "dead," in which fish carcasses were collected from the river or laboratory. Kappa statistic: <0 = poor agreement; 0–0.2 = slight agreement; 0.21–0.4 = fair agreement; 0.4–0.61 = moderate agreement; 0.61–0.8 = substantial agreement.[31]

Renibacterium salmoninarum

Histology. Eleven fish were diagnosed as positive for *R. salmoninarum* using histology (Table 1). Focal granulomatous

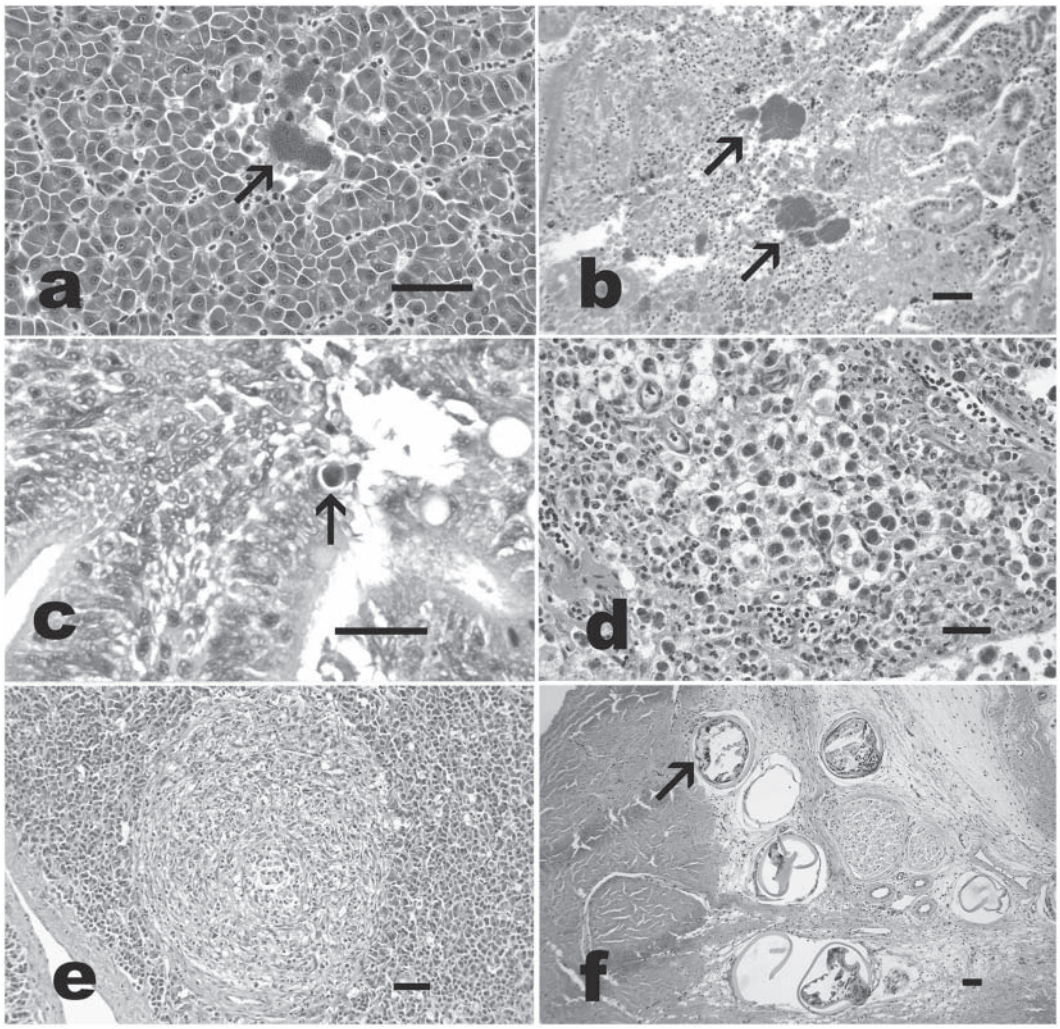

Figure 1. Pathogens observed in healthy and dead adult Chinook salmon (*Oncorhynchus tshawytscha*). Hematoxylin and eosin. Bars = 50 μm. **a**, *Aeromonas salmonicida* (arrow) in the liver of an asymptomatic fish that was euthanized; **b**, *A. salmonicida* colonies (arrows) in kidney from a dead fish collected from the field; **c**, early, light infection by *Ceratomyxa shasta* presporogenic form (arrow) in the gut epithelium; **d**, massive infection of *C. shasta* presporogenic stages in the pyloric caeca, in which spores were not detected by wet mount; **e**, granuloma in the liver of a fish collected live that diagnosed positive for *Renibacterium salmoninarum*; fish was negative by enzyme-linked immunosorbent assay and direct fluorescent antibody test of kidney tissue; **f**, metacercariae of *Nanophyetus salmincola* (arrow) in the base of gill.

inflammatory lesions were observed in kidney, spleen, or liver (Fig. 1e). Several of the infected fish exhibited the infection in only the spleen or liver. In severe infections, the lesions coalesced within the kidney interstitium, resulting in severe, locally extensive, chronic interstitial nephritis.

Comparison. A total of 111 fish were available that had both complete histology and ELISA values, including both live and dead fish (Table 1). The overall agreement between histology and the 2 approved screening tests for *R. salmoninarum* was low (Table 2). The agreement between test results when fish were classified as positive by ELISA was very low (sensitivity = 16%); however, most of the ELISA-positive fish that tested negative by histology only had low ELISA optical density (OD) readings (0.100–0.2000). Shifting the positive cutoff threshold to increase the OD reading for positives improved the sensitivity of histology slightly to 23.5%. The ability of histology to detect *R. salmoninarum* lesions was slightly better when results were compared to those of the DFAT (Table 2), but the 95% confidence intervals overlapped, suggesting the difference was not statistically significant.

Interestingly, there was very poor agreement (kappa = 0.290) between the ELISA and DFAT on the subgroup of samples evaluated using these 2 approved tests. Agreement was improved (kappa = 0.615) when light DFAT infections (<1 bacteria per 1,000× field) were reclassified as negative because the disagreement between the tests was mostly due

to samples that tested low positive on DFAT and negative on the ELISA. Despite the stricter DFAT cutoff, 4 out of 18 negative ELISA tests were still positive on DFAT. The DFAT results for fish that were histology positive and ELISA negative was of particular interest. Here, 2 of the 6 fish were also negative by DFAT. Both fish were diagnosed as positive for *R. salmoninarum* based on observation of granulomas in the liver, which were absent in the kidney and spleen (Fig. 1e). Direct FATs and ELISA are usually run on kidney tissues.

Ceratomyxa shasta

Histology. Some fish exhibited infections ranging from light (presumably early) infections, in which few trophozoites were observed in the intestinal epithelium, to moderate to heavy infections, which were characterized by numerous trophozoites (presporogenic forms) occurring throughout all layers of the gut and occasionally extending through the muscularis. Extraintestinal infections were also observed in a few fish with intestinal infections, which were characterized by focal infections, usually in the liver, in which numerous trophozoites and spores were associated with severe necrosis and chronic inflammation. The intestinal epithelium is one of the first tissues to exhibit postmortem autolysis. Interestingly, in these samples, intact *C. shasta* trophozoites were often observed among host tissue that was essentially dissolved by postmortem autolysis.

Comparison. A total of 136 (108 live and 28 dead) fish were examined (Table 1). The proportion of wet mount–positive fish that tested positive by histology was high (71–86%), depending on whether the fish were collected alive or dead (Table 2). However, between 28% and 63% of the fish negative by wet mounts were positive by histology (Table 2).

Nanophyetus salmincola

Histology. Metacercariae consistent with *N. salmincola* were found in essentially all organs, particularly the kidney and gills (Fig. 1f). These parasites were most numerous in the kidney.

Comparison. A total of 229 (149 live and 80 dead) were examined for *N. salmincola*, and most of the fish were positive for this parasite by wet mounts of the kidneys. Many of the positive fish by wet mount were also positive by histology regardless of whether euthanized subclinical fish or dead fish were examined (Table 2). Kappa scores were poor to fair (<0.4). The 33 fish that were negative by histology but positive by wet mounts had light infections (mean: 168 parasites/g kidney; range: 1–1,410). In contrast, fish that were positive by both tests had a mean of 512 (range: 1–7,156) parasites/g. Approximately one-third of the 22 fish that were negative for *N. salmincola* by wet mount were positive by histology (all from the live group; Table 1). Five of these 7 histology-positive fish exhibited infections only in the gills (Fig. 1f). Metacercariae were most often seen within the gill arch at the base of the filament. Wet mounts for this parasite were only conducted on the kidneys, which would explain why the parasites were missed on the standard test.

Selected diagnostic examples

With a total of 259 fish that were evaluated in the current study, it is beyond the scope of this article to describe diagnostic results for each fish. However, the following are examples demonstrating the possibility of misdiagnoses of disease if histology was not employed. One moribund fish was negative for *C. shasta* and positive for *A. salmonicida* using traditional tests, but histological examination revealed severe, invasive ceratomyxosis of the intestine and pyloric cecae (Fig. 1d) and no bacterial colonies in the tissues. Hence, without histology, coinfection with *C. shasta* would not have been detected. Another fish was *R. salmoninarum* positive by the ELISA (OD = 0.1355) and negative for *C. shasta* in wet mounts. Histology revealed no granulomatous lesions consistent with *R. salmoninarum* and profound intestinal infection by *C. shasta*. If histology had not been employed, the primary diagnosis would have been BKD (*R. salmoninarum* infection), and ceratomyxosis would have gone undetected. Bacterial kidney disease in this case was most likely secondary to *C. shasta* based on the pathology. In addition, histological examinations revealed infections by *Parvicapsula minibicornis* in the kidney and *Myxobolus* sp. in the hind brain in many fish. Occasional infections by anisakine nematode larvae in the mesenteries, adult tapeworms (probably *Eubothrium* sp.) in the gut lumen, *Myxidium* sp. in the kidney, *Loma salmonae* in the heart, and *Ichthyophthirius multifiliis* and gill copepods (*Salmincola californiensis*) were also observed.

Discussion

Histology is commonly used in terrestrial animals to aid in the diagnosis of disease. This technique is particularly appropriate when the etiology of a disease is initially unknown[44] or to differentiate between primary and secondary causes of death. In the present study, the use of histology resulted in a different primary diagnosis than what would have been achieved using only the traditional Blue Book tests for a few fish examined. However, data generated from the current study suggest histology will miss subclinical infections with at least 2 common bacterial pathogens found in the Pacific Northwest (Table 2). In general, the ability of histology to identify infected animals was improved when only dead fish were evaluated (Table 2). In these cases, histology also had the benefit of detecting other pathogens and pathology associated with noninfectious agents.

Covert infections with *A. salmonicida* in salmonids have been recognized since the pathogen was described approximately 100 years ago.[24] In the present study, the bacterium was cultured from many of the apparently healthy fish in which it was not easily visualized by histology. It would be expected that culture would be better at detecting *A. salmonicida* infections than histology, as this bacterium grows well on TSA medium, resulting in exponential amplification of the agent. Therefore, the finding that not all fish with a positive culture tested positive by histology was not surprising. Assuming culture was properly identifying infected fish, removing "live fish," which likely had light infections, from the analysis improved the sensitivity of histology, but approximately 40% of the infections were still missed.

A challenge that many fish health professionals face is the rapid decomposition of tissues after death. It is well recognized that culture of pathogenic bacteria from dead fish may be compromised by postmortem colonization of organs by rapidly growing, saprophytic bacteria from the gastrointestinal tract or water.[35] Postmortem colonization of organs by bacteria was of particular concern for diagnosis of *A. salmonicida* by culture because TSA is not a selective medium and thus saprophytes from tissues of dead fish could easily overgrow the culture plates. Conversely, confusion of saprophytic bacteria that had colonized organs postmortem with *A. salmonicida* in histological sections was also a concern. A criterion used to identify postmortem bacteria colonization is the absence of an inflammatory response,[35] and this is precisely the histological presentation of *A. salmonicida* in live fish.[19,24] Nevertheless, the results indicate that postmortem problems did not compromise the ability to properly detect *A. salmonicida* colonies in tissues, and only 6 of 28 histology-positive fish were culture negative. If postmortem saprophytic bacteria were misidentified as *A. salmonicida*, then a decrease in agreement between histology and culture in the dead fish that tested negative on culture would have been expected, but this group actually showed both higher kappa scores and sensitivity (ability of the test to properly classify infected fish; Table 2).

Histology was also poor at detecting *R. salmoninarum* infections compared to the other 2 approved Blue Book tests, particularly when infections occurred in live subclinical fish. *Renibacterium salmoninarum* often presents as a chronic disease, and subclinical infections are prevalent in Chinook salmon throughout the fish's lifecycle.[2,3,28,45,51,52] Therefore, it would be expected that many infected Chinook salmon are asymptomatic and that many of these fish would not exhibit lesions at a histological level. There are 2 studies comparing histology to other tests for *R. salmoninarum* infections. A laboratory transmission study with Chinook salmon showed excellent agreement between DFAT and histology on kidneys.[36] Another study[9] compared various tests for the bacterium, including ELISA, indirect FAT, Gram stain kidney imprints, histology, and PCR from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) from 3 farms. More fish were diagnosed by ELISA and FATs than histology, but results were still comparable. In the present study, agreement between ELISA results and histology was rather poor. Disagreement between histology and ELISA occurred for both positive and negative ELISA results but was particularly poor for ELISA-positive fish (Table 2). The main purpose of ELISA is to screen healthy fish for subclinical infections, providing a ranking of level of infection based on the presence of the p57 bacterial protein.[14–16,40–42] The ELISA is now used extensively in salmon hatcheries in western Canada and the United States to identify brood stock with the least risk for maternal transmission of the bacterium.

Analysis of kidney with the DFAT for *R. salmoninarum* was added to a subset of samples in an attempt to resolve the discrepancies in results between ELISA and histology. When the ELISA and DFAT (both Blue Book–recommended tests for *R. salmoninarum*) were compared, they did not agree very strongly (kappa = 0.29–0.615). Several of the fish that were histology negative but ELISA positive were also positive by DFAT, indicating that for detection of the bacterium, the 2 Blue Book tests are better at detecting infections than histology. Other researchers have found that ELISA is usually more sensitive than DFAT for *R. salmoninarum*,[33] but the 2 tests are correlated.[39] In the current study, bacteria consistent with *R. salmoninarum* were observed in kidney smears stained with DFAT in several fish that were ELISA negative. Moreover, a few fish that were positive by histology and negative by ELISA were also negative by DFAT. Such conflicting results can be explained by the diagnostic endpoints of each test and by the nature of the infection and the associated lesions. Whereas the name of the disease caused by *R. salmoninarum* infection, BKD, refers to an infection primarily in the kidney, fish may exhibit the infection and lesions in other organs, such as the liver[9] or brain,[47] without obvious kidney lesions. In the present study, granulomatous lesions were confined to the liver in both fish that were positive by histology but negative by the 2 other tests (Table 3). The findings of the present study continue to support several other studies comparing different diagnostic tests for this chronic, sometimes organ-specific, infection. Discrepancies in test results are likely due largely to different diagnostic criteria; ELISA evaluates the presence of a soluble antigen distributed throughout the fish, which may persist in recovered fish,[41] DFAT directly visualizes intact bacteria in kidney tissue, and histology identifies the pathological effects of the infection in whichever organ is examined. The current study indicates that none of the tests evaluated were consistent at identifying *R. salmoninarum* infections, so screening with any of them will probably result in misclassification of some positive animals. Which of the tests is more accurate could not be determined in the current study. In recent years, researchers have relied more on PCR tests for

Table 3. Comparisons of histology with enzyme-linked immunosorbent assay (ELISA) and direct fluorescent antibody test (DFAT) results for *Renibacterium salmoninarum* results in live and dead Chinook salmon (*Oncorhynchus tshawytsch*a).

Histology*	Live/Dead†	ELISA optical density‡	DFAT bacteria presence§
K, L, S	D	H, 2.264	++++
K	D	H, 1.5525	+++
K	D	N, 0.062	++
L, S	D	N, 0.0825	++
K S	L	N, 0.0835	+
L	L	N, 0.0680	N
L	L	N, 0.0600	+
L	L	N, 0.0675	N
0	D	H, 0.9135	+
0	D	H, 2.024	+++
0	D	H, 2.125	++++
0	D	M, 0.267	+
0	D	H, 1.5835	+++
0	D	H, 2.2295	++++
0	D	N, 0 .0725	+
0	D	N, 0.0625	+
0	L	N, 0.060	+
0	L	N, 0.060	++
0	L	N, 0.0655	+
0	L	N, 0.0590	N
0	L	N, 0.0600	N
0	D	N, 0.0645	N
0	D	N, 0.0635	N
0	D	N, 0.0675	N

* Organs that were positive for *R. salmoninarum* lesions (i.e., with granulomatous lesions) are listed as follows: K = kidney; S = spleen; L = liver; 0 = no *R. salmoninarum* lesions observed.
† D = dead; L = live.
‡ ELISA results are presented with optical density reading and category score: H = high; M = medium; N = negative.
§ +++ = >100 bacteria/1,000× field; +++ = 50–100 bacteria/1,000× field; ++ = 1–10 bacteria/1,000× field; + = bacteria present, but <1/1,000× field; N = negative, no *R. salmoninarum* bacteria observed.

surveys of *R. salmoninarum*,[2,43] which may be more sensitive than any of the 3 methods used in the present study.

Despite histology not being as efficient at detecting subclinical bacterial infections with *R. salmoninarum* and *A. salmonicida*, it may be more effective than wet mounts at detecting parasitic infections with *C. shasta* and *N. salmonicida* (Table 2). Usually direct examination of wet tissues is more sensitive than histology for detecting myxozoans,[22] but this was not the case in the current study. For *C. shasta*, the presence of spores is usually required for detection by the wet mount method because presporogenic stages are difficult to visualize. Exposure studies conducted with juvenile fish showed that progression of infection of *C. shasta* and associated morbidity is temperature dependent,[23] and spores can be seen as soon as 2 weeks after infection at 18°C, while 55 days may be required at 12°C.[49] Hence, at least with juvenile fish, detection of spores should correlate with significant infections after a few weeks during the summer months in the Willamette River system. Many fish were negative for *C. shasta* by wet mount but positive by histology, and some of these fish had severe intestinal infections. Close examination of the histological slides from these fish usually revealed very few or no spores, which may explain the discrepancy between the test results. The absence of spores in heavily infected fish was observed in fish collected in both the summer and fall, indicating that these fish were likely exposed to the infectious stage of the parasite over many months. Perhaps the progression of the infection in adult salmon differs from juveniles, with massive proliferation occurring for many weeks without significant sporulation.

Sensitivity of histology compared to wet mounts for *N. salmincola* was greater than 70%. When histology failed to detect infected fish as determined by wet mount, it was almost always with light infections. Metacercariae most often cause disease in fish only in heavy infections,[37] and mortality associated with *N. salmincola* in salmon is correlated with intensity of infection.[12,20,25] It would therefore be unlikely that missing these light infections by histology would have compromised diagnoses for cause of morbidity. Although some light infections appeared to only be detected by wet mount, several infections were detected by histology and not this method. It is very unlikely that the infection by histology was misdiagnosed because the metacercariae of *N. salmincola* have a very distinct morphology and have been well characterized by wet mounts[21] and histology.[10,22,44] Interestingly, 5 of the 7 fish that were histology positive and wet mount negative exhibited infections only in the gills, which were not evaluated by wet mount. Whereas the posterior kidney is a primary site of infection by this parasite, metacercariae of *N. salmincola* are found in essentially every organ.[44] Perhaps the distribution of the infection is reflected by route of entry, as cercariae enter the body via the gills, skin, or even the gut if infected snails are eaten.[17] If gill wet mounts were not included in a diagnostic evaluation, then fish infected with these localized infections would be misdiagnosed.

In the case of both parasites evaluated herein, it appears that some parasite infections were not detected with the standard wet mount evaluation. This suggests that replacing organ wet mounts with histology may be more accurate for detecting these types of infections, even when the animals are not clinically diseased. Although some light infections would be missed by histology, results of the current study suggest that wet mounts miss more infections.

Histology also has the advantage in that it detects the presence and relative abundance of numerous parasites not screened for by the Blue Book methods approach, and several other parasites were detected in the current study with this method. Of these, *P. minibicornis* would not have been readily detected by wet mounts as its spores are very small and many infections are characterized by presporogenic

forms.[29] This parasite was seen in the renal glomeruli and tubules of many fish and has been associated with PSM in sockeye salmon.[8]

In conclusion, although not all positive animals were detected by histology for any of the pathogens that were examined (agreement was never 100%), histology may actually detect some parasitic infections better than the Blue Book–recommended methods. Furthermore, other parasites and lesions were observed that would only be detected by histology. Because it appears that the level of a pathogen in the host needs to be relatively high before histology is considered sensitive (as apparent in the differences between the ability of histology to detect pathogens in dead vs. live fish), replacing the Blue Book standard tests with histology is not recommended, but rather it should be added to the screening program. Histology continues to be the primary diagnostic method in the study of PSM in the Willamette system. Adding histology as a diagnostic tool in screening programs and interpreting the results in parallel (positive on either test results in a positive classification) could improve the overall sensitivity of screening programs. Moreover, this method also can be very useful for the detection of other (emerging or unsuspected) pathogens[44,45] as most microbiological assays do not detect the presence or severity of pathogens for which the test is not designed for. The drawback is the added cost; however, this could be curtailed by conducting histology on only a subset of individuals. Results presented herein suggest that histology on the moribund fish from a population may be of greater value than the subclinical fish. It should be mentioned that although both clinically diseased and subclinical fish were evaluated, the study was restricted to 1 salmon species and 1 watershed. Extrapolating to other species in other geographic areas, therefore, should be done with caution, particularly if the data are based on other diagnostic methods.

Acknowledgements

The authors thank the staff of Oregon Department of Fish and Wildlife for assistance with this study and conducting bacterial kidney disease (BKD) assays; Ms. Leslie Lindsay conducted the enzyme-linked immunosorbent assay (ELISA) tests and Mr. Craig Banner conducted the direct fluorescent antibody test (DFAT) test. The authors also thank Dr. Chris Caudill and his staff (University of Idaho) for assistance with collecting samples and Drs. Jerry Heidel, Stephen Feist, and Justin Sanders for manuscript review.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was funded in part by the U.S. Army Corps of Engineers.

References

1. American Fisheries Society–Fish Health Section (AFS-FHS): 2010, AFS-FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2010 ed. AFS-FHS, Bethesda, MD.
2. Arkoosh MR, Clemons E, Kagley AN, et al.: 2004, Survey of pathogens in juvenile salmon *Oncorhynchus* spp. migrating through Pacific Northwest estuaries. J Aquat Anim Health 16:186–196.
3. Banner CR, Long JJ, Fryer JL, Rohovec JS: 1986, Occurrence of salmonid fish infected with *Renibacterium salmoninarum* in the Pacific Ocean. J Fish Dis 9:273–275.
4. Banner CR, Rohovec JS, Fryer JL: 1982, A rapid method for labeling rabbit immunoglobulin with fluorescein for use in detection of fish pathogens. Bull Eur Assoc Fish Pathol 2:35–37.
5. Bartholomew JL: 2002, Salmonid ceratomyxosis. *In*: American Fisheries Society–Fish Health Section (AFS-FHS) blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens. AFS-FHS, Bethesda, MD.
6. Bartholomew JL, Ray E, Torell B, et al.: 2004, Monitoring *Ceratomyxa shasta* infection during a hatchery rearing cycle: comparison of molecular, serological and histological methods. Dis Aquat Organ 62:85–92.
7. Blazer VS, Iwanowicz LR, Starliper CE, et al.: 2010, Mortality of centrarchid fishes in the Potomac drainage: survey results and overview of potential contributing factors. J Aquat Anim Health 22:190–218.
8. Bradford MJ, Lovy J, Patterson DA, et al.: 2010, *Parvicapsula minibicornis* infections in gill and kidney and the premature mortality of adult sockeye salmon (*Oncorhynchus nerka*) from Cultus Lake, British Columbia. Can J Fish Aquat Sci 67:673–683.
9. Bruno D, Collet B, Turnbull A, et al.: 2007, Evaluation and development of diagnostic methods for *Renibacterium salmoninarum* causing bacterial kidney disease (BKD) in the UK. Aquaculture 269:114–122.
10. Bruno DW, Nowak B, Elliot DG: 2006, Guide to the identification of fish protozoan and metazoan parasites in stained tissue sections. Dis Aquat Organ 70:1–36.
11. Bruno DW, Poppe TT: 1996, A colour atlas of salmonid diseases. Academic Press, New York, NY.
12. Butler JA, Millemann RE: 1971, Effect of the "salmon poisoning" trematode, *Nanophyetus salmincola*, on swimming ability of juvenile salmonid fishes. J Parasitol 57:860–865.
13. Eaton WD, Kent ML, Meyers TR: 1991, Coccidia, X-cell pseudotumors and *Ichthyophonus* sp. infections in walleye pollock (*Theregra chalcogramma*) from Auke Bay, Alaska. J Wildl Dis 27:140–143.
14. Elliot DG, Pascho RJ, Bullock GL: 1989, Developments in the control of bacterial kidney disease of salmonid fishes. Dis Aquat Org 6:201–215.
15. Elliot DG, Pascho RJ, Jackson LM, et al.: 1997, *Renibacterium salmoninarum* in spring-summer Chinook salmon smolts at

dams on the Columbia and Snake Rivers. J Aquat Anim Health 9:114–126.
16. Elliot DG, Pascho RJ, Palmisano AN: 1995, Brood stock segregation for the control of bacterial kidney disease can affect mortality of progeny Chinook salmon (*Oncorhynchus tshawytscha*) in seawater. Aquaculture 132:133–144.
17. Farrell RK, Lloyd MA, Earp B: 1964, Persistence of *Neorickettsiae helminthoeca* in an endoparasite of the Pacific salmon. Science 145:162–163.
18. Feist SW, Longshaw M: 2008, Histopathology of fish parasite infections—importance for populations. J Fish Biol 73:2143–2160.
19. Ferguson HW: 2006, Kidney. *In*: Systemic pathology of fish, pp. 91–118. Scotia Press, London, UK.
20. Ferguson JA, Koketsu W, Ninomiya I, et al.: 2011, Mortality of coho salmon (*Oncorhynchus kisutch*) associated with burdens of multiple parasite species. Int J Parasitol 41:1197–11205.
21. Ferguson JA, Schreck CB, Chitwood R, Kent ML: 2010, Persistence of infection by metacercariae of *Apophallus* sp., *Neascus* sp., and *Nanophyetus salmincola* plus two myxozoans (*Myxobolus insidiosus* and *Myxobolus fryeri*) in coho salmon (*Oncorhynchus kisutch*). J Parasitol 96:340–347.
22. Ferguson JA, St-Hilaire S, Peterson TS, et al.: 2011, Survey of parasites in threatened stocks of coho salmon (*Oncorhynchus kisutch*) in Oregon by examination of wet tissues and histology. J Parasitol 97:1085–1098.
23. Hallett SL, Bartholomew JL: 2012, *Myxobolus cerebralis* and *Ceratomyxa shasta. In*: Fish parasites: pathobiology and protection, ed. Woo PTK, Buchmann K, pp. 131–162. CABI, Oxfordshire, UK.
24. Hiney M, Olivier G: 1999, Furunculosis. *In*: Fish diseases and disorders, vol. 3: viral, bacterial and fungal pathogens, ed. Woo PTK, Bruno DW, pp. 341–425. CABI, Oxfordshire, UK.
25. Jacobson KC, Teel D, Van Doornik DM, Castillas E: 2008, Parasite-associated mortality of juvenile Pacific salmon caused by the trematode *Nanophyetus salmincola* during early marine residence. Mar Ecol Prog Ser 354:235–244.
26. Keefer ML, Taylor GA, Garletts DF, et al.: 2010, Prespawn mortality in adult spring Chinook salmon outplanted above barrier dams. Ecol Freshwater Fish 19:361–372.
27. Kent ML, Poppe TP: 1998, Bacterial diseases. *In*: Diseases of seawater netpen-reared salmonids, pp. 17–35. Pacific Biological Station Press, Nanaimo, British Columbia, Canada.
28. Kent ML, Traxler GS, Kieser D, et al.: 1998, Survey of salmonid pathogens in ocean-caught fishes in British Columbia, Canada. J Aquat Anim Health 10:211–219.
29. Kent ML, Whitaker DJ, Dawe SC: 1997, *Parvicapsula minibicornis* n. sp. (Myxozoa, Myxosporea) from the kidney of sockeye salmon (*Oncorhynchus nerka*) from British Columbia, Canada. J Parasitol 83:1153–1156.
30. Koponen K, Myers MS, Ritola O, et al.: 2001, Histopathology of feral fish from a PCB-contaminated freshwater lake. Ambio 30:122–126.
31. Landis JR, Koch GG: 1977, The measurement of observer agreement for categorical data. Biometrics 33:159–174.
32. Longshaw M, Frear PA, Nunn AD, et al.: 2010, The influence of parasitism on fish population success. Fish Management Ecol 17:426–434.
33. Meyers TR, Short S, Farrington C, et al.: 1993, Comparison of the enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody test (FAT) for measuring the prevalences and levels of *Renibacterium salmoninarum* in wild and hatchery stocks of salmonid fishes in Alaska, USA. Dis Aquat Org 16:181–189.
34. Millemann RE, Knapp SE: 1970, Pathogenicity of the "salmon poisoning" trematode, *Nanophyetus salmincola*, to fish. *In*: A symposium on diseases of fishes and shellfishes, ed. Snieszko SF, pp. 209–217. American Fisheries Society, Bethesda, MD.
35. Morris JA, Harrison LM, Partridge SM: 2007, Practical and theoretical aspects of postmortem bacteriology. Curr Diagn Pathol 13:65–74.
36. O'Farrell CL, Elliott DG, Landolt ML: 2000, Mortality and kidney histopathology of Chinook salmon *Oncorhynchus tshawytscha* exposed to virulent and attenuated *Renibacterium salmoninarum* strains. Dis Aquat Organ 43:199–209.
37. Paperna I, Dzikowski R: 2006, Digenea (phylum Platyhelminthes). *In*: Fish diseases and disorders, vol. 1: protozoan and metazoan infections, ed. Woo PTK, pp. 345–390. CABI, Oxfordshire, UK.
38. Pascho RJ, Elliot DG: 2003, Bacterial kidney disease. *In*: American Fisheries Society–Fish Health Section blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens. AFS-FHS, Bethesda, MD.
39. Pascho RJ, Elliot DG, Mallet RW, Mulcahy D: 1987, Comparison of five techniques for the detection of *Renibacterium salmoninarum* in adult coho salmon. Trans Am Fish Soc 116:882–890.
40. Pascho RJ, Elliot DG, Streufert JM: 1991, Brood stock segregation of spring Chinook salmon *Oncorhynchus tshawytscha* by use of the enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody technique (FAT) affects the prevalence and levels of *Renibacterium salmoninarum* infection in progeny. Dis Aquat Organ 12:25–40.
41. Pascho RJ, Goodrich TD, McKibben CL: 1997, Evaluation by enzyme-linked immunosorbent assay (ELISA) of *Renibacterium salmoninarum* bacterins affected by persistence of bacterial antigens. J Aquat Anim Health 9:99–107.
42. Pascho RJ, Mulcahy D: 1987, Enzyme-linked immunosorbent assay for a soluble antigen of *Renibacterium salmoninarum*, the causative agent of salmonid bacterial kidney disease. Can J Fish Aquat Sci 44:183–191.
43. Rhodes LD, Durkin C, Nance SL, Rice CA: 2006, Prevalence and analysis of *Renibacterium salmoninarum* infection among juvenile Chinook salmon *Oncorhynchus tshawytscha* in North Puget Sound. Dis Aquat Organ 71:179–190.
44. Rodnick KJ, St-Hilaire S, Battiprolu PK, et al.: 2008, Evidence for sex and or habitat differences in swimming performance, parasite infestation, tissue biochemistry, and morphology of

juvenile coho salmon in Oregon's West Fork Smith River. Trans Am Fish Soc 137:1571–1590.
45. Saksida S, Marty GD, Jones SR, et al.: 2012, Parasites and hepatic lesions among pink salmon, *Oncorhynchus gorbuscha* (Walbaum), during early seawater residence. J Fish Dis 35:137–151.
46. Scholz NL, Myers MS, McCarthy SG, et al.: 2011, Recurrent die-offs of adult coho salmon returning to spawn in Puget Sound lowland urban streams. PLoS One 6:e28013.
47. Speare DJ: 1997, Differences in patterns of meningoencephalitis due to bacterial kidney disease in farmed Atlantic and Chinook salmon. Res Vet Sci 62:79–80.
48. Stentiford GD, Longshaw M, Lyons BP, et al.: 2003, Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Mar Environ Res 55:137–159.
49. Udey LR, Fryer JL, Pilcher KS: 1975, Relation of water temperature to ceratomyxosis in rainbow trout (*Salmo gairdneri*) and coho salmon (*Oncorhynchus kisutch*). J Fish Res Board Can 32:1545–1551.
50. U.S. Fish and Wildlife Service, American Fisheries Society–Fish Health Section (AFS-FHS): 2010, Standard procedures for aquatic animal health inspections. *In*: AFS-FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2010 ed. AFS-FHS, Bethesda, MD.
51. Van Gaest AL, Dietrich JP, Thompson DE, et al.: 2011, Survey of pathogens in hatchery Chinook salmon with different outmigration histories through the Snake and Columbia Rivers. J Aquat Anim Health 23:62–77.
52. Weins GD: 2011, Bacterial kidney disease (*Renibacterium salmoninarum*). *In*: Fish diseases and disorders, vol. 3: viral, bacterial and fungal infections, ed. Woo PTK, Bruno DW, pp. 338–375. CABI, Oxfordshire, UK.
53. World Organization for Animal Health (OIE): 2003, OIE manual of diagnostic tests for aquatic animals. OIE, Paris, France.