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DIAGNOSTIC METHODOLOGY IS CRITICAL FOR ACCURATELY DETERMINING THE PREVALENCE OF *ICHTHYOPHONUS* INFECTIONS IN WILD FISH POPULATIONS

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ABSTRACT: Several different techniques have been employed to detect and identify *Ichthyophonus* spp. in infected fish hosts; these include macroscopic observation, microscopic examination of tissue squashes, histological evaluation, in vitro culture, and molecular techniques. Examination of the peer-reviewed literature revealed that when more than 1 diagnostic method is used, they often result in significantly different results; for example, when in vitro culture was used to identify infected trout in an experimentally exposed population, 98.7% of infected trout were detected, but when standard histology was used to confirm known infected tissues from wild salmon, it detected ~50% of low-intensity infections and ~85% of high-intensity infections. Other studies on different species reported similar differences. When we examined a possible mechanism to explain the disparity between different diagnostic techniques, we observed non-random distribution of the parasite in 3-dimensionally visualized tissue sections from infected hosts, thus providing a possible explanation for the different sensitivities of commonly used diagnostic techniques. Based on experimental evidence and a review of the peer-reviewed literature, we have concluded that in vitro culture is currently the most accurate diagnostic technique for determining infection prevalence of *Ichthyophonus*, particularly when the exposure history of the population is not known.

Epidemiologists rely on accurate assessments of both prevalence and intensity of infection to establish patterns of morbidity and mortality within populations. To do this, they employ sampling methods that accurately assess the number of mortalities, as well as susceptible, infected, diseased, and recovered individuals in a population (Gordis 2000); the methods used vary with the pathogen being studied and the logistics of sample collection.

A number of field studies have capitalized on the presence of *Ichthyophonus* sp. in different tissues of the piscine host to identify infected individuals, as well as establish and track infection prevalence in wild populations. These studies employed several methods of pathogen identification, including macroscopic examination of tissues (Anonymous 1993), microscopic visualization of tissue squashes (Holst, 1994; Rahimian and Thulin, 1996), histological evaluation (Marty et al., 1998; Kent et al., 2001; Conboy and Speare, 2002; Jones and Dawe, 2002), in vitro culture of tissue explants (Hershberger et al., 2002; Kocan et al., 2004; Halos et al., 2005), and polymerase chain reaction (PCR) using *Ichthyophonus*-specific primers (Whipps et al., 2006). In those studies using more than 1 method for evaluating infection prevalence, it became apparent that different diagnostic methods produce significantly different results within the same population and even within the same individual. For example, microscopic examination of tissue squash preparations often detects higher infection prevalence than does macroscopic examination of the same tissues (Holst, 1994; Rahimian and Thulin, 1996). Likewise, 70% (21/30) of adult wild Pacific herring (*Clupea pallasii*) were positive for *Ichthyophonus* sp. by in vitro culture, but only 7% (2/30) of the same fish were positive by conventional histological examination of heart, liver, and spleen tissue (Kocan et al., 1999). The same authors reported a similar discrepancy between in vitro culture and histological values in experimentally infected herring where 100% were culture-positive, but only 10% were histologically positive. In a different study using a different species, 11%

(33/302) of Puget Sound rockfish (*Sebastes emphaeus*) were positive by in vitro culture, while less than 1% (1/302) of the same fish were positive by histology in any tissue, and 0% exhibited visible lesions (Halos et al., 2005). Using culture-positive and/or histologically positive cardiac tissue from infected Chinook salmon (*Oncorhynchus tshawytscha*) as reference samples, Whipps et al. (2006) detected between 43% and 50% of confirmed positive tissues by PCR and stated “light infection of *Ichthyophonus hoferi* was not detected as often as heavy infection when using PCR on a single tissue.”

All of the above studies demonstrate a significant difference between various diagnostic methods to accurately detect *Ichthyophonus* spp.-infected fish. Because of the discrepancies in parasite detection sensitivity and the importance of obtaining accurate prevalence data for epizootiologic studies, we attempted to experimentally determine whether the reported discrepancies were real and, if so, what underlying factors result in underestimates of infected fish. As a model for evaluating the reported discrepancies, we chose in vitro explant culture and histological evaluation, 2 frequently used techniques that directly visualize the parasite in situ. To help explain what mechanism is responsible for the variable results, we used 3-dimensional (3-D) visualization of the distribution of the parasite in herring skeletal muscle.

MATERIALS AND METHODS

To evaluate the ability of in vitro explant culture to accurately detect *Ichthyophonus* spp.-infected fish, 3 groups of rainbow trout (*Oncorhynchus mykiss*) totaling 130 fish were experimentally exposed to *Ichthyophonus* sp., then sub-sampled 30 days post-exposure and examined by in vitro culture for the presence of *Ichthyophonus* sp. The fish were exposed by feeding minced tissues from infected trout twice over a 24-hr period, then maintained in flowing spring water at 15 C and fed commercial trout chow for 30 days, after which they were killed with an overdose of buffered tricaine methane-sulfonate (MS-222) and necropsied. A second unexposed group of 130 fish served as controls. During necropsy, the heart was aseptically removed from a sub-sample of fish ($n = 76$) from both exposed and control groups and cultured in Eagles Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum and antibiotics (McVicar, 1982; Spanggaard et al., 1994; Kocan et al., 2004), incubated at 15 C for 21 days, then examined under 40× magnification for growth of *Ichthyophonus* sp.

To experimentally evaluate the sensitivity of standard histological evaluation to detect known *Ichthyophonus* spp.-infected tissues, we retrieved formalin-preserved archived tissues of Chinook salmon known to be *Ichthyophonus* sp.-positive as determined by in vitro culture (Kocan

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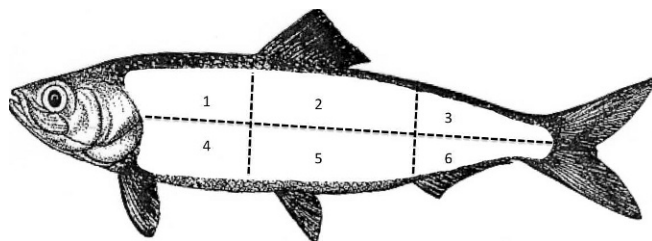


FIGURE 1. Sector numbering system used for evaluating epidermal ulcers and *Ichthyophonus* cells in underlying skeletal muscle.

et al., 2004; Kocan and Hershberger, 2006), and re-evaluated them using conventional single plane histology. Two sets of naturally infected positive tissues were used. The first tissue set ($n = 42$) was collected in 2002 and 2003 from Chinook salmon at the mouth of the Yukon River when the infection was at its earliest stages and the fish exhibited minimal clinical signs (Kocan et al., 2004). A second set of positive tissues ($n = 62$) was collected between river-km 1,104 and 5,588 after the infection had progressed for an additional 2–3 wk and was clinically visible and disseminated to most tissues.

Because the heart is the primary target organ for *Ichthyophonus* sp. in salmonids, clupeids, and cottids (Rand and Cone, 1990; Jones and Dawe, 2002; Kocan et al., 2004) and is often the only infected organ, it was used as the reference tissue for comparing the sensitivity of histology to in vitro culture. A 0.5-cm thick transverse section of tissue was removed from the apex of each infected heart and discarded; a second similar section was placed into 10% neutral buffered formalin for 48 hr, transferred to 70% ethanol, trimmed, and finally placed into standard tissue cassettes for routine histological processing. After embedding in paraffin blocks, 5- μ m sections were cut on a rotary microtome (American Optical, Buffalo, New York) and transferred to charged glass slides (Probe-On™, Fisher Scientific, Pittsburgh, Pennsylvania). Although *Ichthyophonus* sp. has neither chitin nor cellulose, it does stain positive (red-violet) with periodic acid Schiff (PAS) reagent because of the presence of polysaccharides (Rand, 1990). Tissues were considered histologically positive for *Ichthyophonus* sp. if they were morphologically consistent with previously published descriptions and were PAS-positive.

To determine whether the distribution of the parasite within the host tissues influenced the ability of different techniques to detect the organism, we used an established tissue clearing method (Potthoff, 1984), in combination with histochemical staining, to obtain a 3-dimensional view of the parasite in situ, as well as determine the density of the parasite per g of muscle tissue.

Wild Pacific herring were collected by gillnet from Cherry Point (Puget Sound), Washington. The entire heart was removed from each fish and cultured for 14 days in Eagle's Minimum Essential Medium (MEM) + 5% fetal bovine serum and antibiotics to identify *Ichthyophonus* spp.-infected individuals. From the culture-positive individuals 28 males (197 ± 32.4 mm) and 41 females (197 ± 41.5 mm) exhibiting visible dermal ulcers, a clinical sign of *Ichthyophonus* sp., were selected for the study. Both sexes were included to avoid possible sex bias, not for comparative purposes. The left side of each fish was then divided into 6 sectors, with the lateral line serving as the dorsal–ventral division. One vertical line was drawn from the forward most point of the dorsal fin and a second line up from the forward most point of the anal fin, producing a pattern of 6 sectors on the surface of each fish (Fig. 1). The mean number and range of epidermal ulcers per sector was determined and analyzed with a 2-factor ANOVA (individual fish and ulcers per sector) to determine whether there was a difference in the distribution of ulcers among sectors as previously reported for infected Atlantic herring (Sindermann and Scattergood, 1954; Rahimian and Thulin, 1996).

To evaluate parasite density in skeletal muscle, a 5-mm wide slice of muscle was cut from the middle of each sector and weighed to the nearest 0.01 g. The fixed muscle tissue was cleared using a modification of Potthoff (1984) by eliminating the steps for staining of bone and cartilage, but retaining the tissue clearing steps. The muscle was then stained with PAS-Schiff reagent (Sigma-Aldrich, St. Louis, Missouri) to visualize *Ichthyophonus* sp. schizonts in the muscle. The total number of PAS-positive *Ichthyophonus* sp. cells, as well as their distribution, was determined for each of the muscle tissue slices under 40 \times magnification,

TABLE I. Detection of infected fish by in vitro culture following experimental per os exposure to *Ichthyophonus*-infected tissue.

Exposure	Number				99% Confidence interval
	N^*	n^\dagger	Positive	%	
1	30	10	10	100	0.0
2	40	36	35	97	7.3
3	60	30	30	100	0.0
Total	130	76	75	98.7	3.3
Control					
1	30	10	0	0	n/a
2	40	36	0	0	n/a
3	60	30	0	0	n/a
Total	130	76	0	0	n/a

* Population exposed.

† Number cultured.

then divided by the weight of the tissue sample to calculate the number of *Ichthyophonus* sp. cells per gram of muscle. The number of epidermal ulcers from each skin sector was then compared with the density of schizonts in the underlying muscle tissue.

RESULTS

In vitro detection of *Ichthyophonus* sp. in experimentally infected trout

Of 76 trout sub-sampled from 130 experimentally exposed fish, 98.7% (75/76) cultured positive for *Ichthyophonus* sp. (Table I). Two sub-samples were 100% culture-positive (30/30 and 10/10), and 1 was 97% culture-positive (35/36). None of the 76 unexposed control fish cultured positive for *Ichthyophonus* sp. after 21 days of incubation.

Histological evaluation

Single plane histology of 5- μ m sections of cardiac muscle detected 74% (77/104) of all culture-confirmed *Ichthyophonus* spp.-infected cardiac tissue from Chinook salmon. Early infections were detected in 55% of known infected tissues, while advanced infections (2- to 3-wk-old) were detected in 87% of known positive tissues (Table II). There was no difference in detection rate between 2002 and 2003, the 2 sample years (Fisher's Exact test; $P = 0.441$ and 0.476), but there was a difference between samples collected early and late in the salmon migration ($\alpha = 0.10$; Fisher Exact test; $P = 0.096$).

Ulcer distribution in skin versus schizont density in underlying skeletal muscle

Epidermal ulcers were not uniformly distributed over the surface of the skin of infected herring, with sector 5 having the highest number of ulcers in both males and females. The mean number of ulcers per sector in all fish ranged from 3.3 to 26.3 (Table III). Similarly, females had a higher mean density of schizonts per sector (160) in skeletal muscle compared with males (92), with the mean number of schizonts per sector for all fish ranging from 58 in sector 6 to 704 in sector 3 (Table IV).

A 3-D visualization of muscle schizont distribution in situ from 4 herring reveal the range of parasite distribution seen in *Ichthyophonus*-infected herring with advanced ichthyophoniasis

TABLE II. Correspondence between culture-positive *Ichthyophonus*-infected Chinook salmon tissues and single plane histology.

Year	N*	Histology positive	% Correspondence
Early infections			
2002	15	7	47
2003	27	16	59
Total	42	23	55
Advanced infections			
2002	25	23	92
2003	37	31	84
Total	62	54	87
Grand total 2002 + 2003	104	77	74

* Number of *Ichthyophonus*-infected samples confirmed by in vitro culture.

(Fig. 2). There was a strong correlation between total epidermal ulcers and total schizont density in the skeletal muscle ($R^2 = 0.759$; $P < 0.01$), but the ulcers in individual sectors did not reflect the number of schizonts in the underlying muscle (Fig. 3).

DISCUSSION

Rationale

McVicar (1999) observed, "there are serious difficulties in determining the true prevalence of (*Ichthyophonus*) infection in natural populations." He postulated that this was due to non-uniform distribution of the parasite between geographic areas and within populations but did not consider the development and distribution of the parasite within the host. Because epidemiological studies rely heavily on accurate infection/disease prevalence data, we attempted to compare several techniques for their suitability to accurately detect *Ichthyophonus* sp. infection in wild populations.

In vitro detection of experimentally infected trout

Similar to previously published studies, we demonstrated that in vitro explant culture is highly accurate in detecting *Ichthyophonus* sp. infections. Because exposure history, duration of infection, and immunologic status of wild fish are unknown, it is difficult to evaluate the effectiveness of any technique to detect infections unless it has been previously tested using hosts of known infection/exposure history. By experimentally exposing

TABLE III. Mean and range of epidermal ulcer density from *Ichthyophonus*-infected Pacific herring (left side).

Sector	Females (n = 41)			Males (n = 28)			Total (n = 69)		
	Mean	Low	High	Mean	Low	High	Mean	Low	High
1	7.0	0	28	1.2	0	7	4.1	0	28
2	10.6	0	42	6.7	0	56	8.6	0	56
3	5.3	0	14	1.4	0	10	3.3	0	14
4	20.8	0	52	3.5	0	13	12.1	0	52
5	37.3	0	104	15.3	0	95	26.3	0	104
6	7.2	0	25	3.4	0	21	5.3	0	25
Mean	14.7	0.0	44.2	5.2	0.0	33.7	9.97	0.0	38.9

TABLE IV. Mean and range of *Ichthyophonus* schizont density (cells/g) in herring skeletal muscle (left side).

Sector	Females (n = 41)			Males (n = 28)			Total (n = 69)		
	Mean	Low	High	Mean	Low	High	Mean	Low	High
1	199	0	1,092	242	0	668	212	0	1,092
2	110	7	460	25	0	62	85	0	460
3	300	73	3,622	82	0	496	704	0	3,622
4	168	2	349	86	0	509	123	0	509
5	94	0	287	98	0	445	96	0	445
6	89	0	186	21	0	100	58	0	186
Mean	160	14	999	92	0	380	213	0	998

non-immune rainbow trout via a natural route (oral), then sub-sampling and examining the fish, we were able to identify 98.7% of the infections (75/76) using in vitro culture. Because in any infected population some individuals will become subclinical carriers, others will become clinical cases, and some will escape infection entirely, it is highly probable that the single culture-negative fish we observed simply did not become infected when exposed. However, even if the culture method failed in this one case, the accuracy was very close to the expected true infection prevalence.

An advantage of in vitro culture is that it allows a live *Ichthyophonus* sp. cell to reproduce during incubation, thus significantly increasing the probability of detection.

Histological evaluation

In the present study, we used known *Ichthyophonus* spp.-infected tissues and determined the accuracy of standard single plane histology to identify infected individuals. Similar to earlier reports, histological evaluation underestimated a significant proportion of known infected tissues, and the technique was especially poor at detecting low-level infections. The sensitivity of histological evaluation increased as infection progressed in a naturally infected population of Chinook salmon, identifying 55% of early infections and 87% after the infection had progressed for several weeks (Table II).

Histological evaluation uses a section of tissue approximately 5- μ m thick, while explant culture uses up to 0.5 g of tissue (2,000–5,000 \times greater mass), thus significantly increasing the probability of detecting low-level or pre-patent infections.

Parasite distribution in skin versus schizont density in skeletal muscle

We hypothesized that the differences observed between in vitro culture and histological evaluation of the same tissues might result from uneven distribution of the parasite within the host tissues. To test this notion, we examined the parasite in situ in herring skeletal muscle. Fish used for comparing parasite distribution within the host were selected by the presence of ulcers on their epidermis, an external clinical sign of *Ichthyophonus* sp. infection (Sindermann and Scattergood, 1954; Rahimian and Thulin, 1996; Hodneland et al., 1997), which typically occurs late in the infection and has been referred to as "sandpaper skin." A wide range of variability was detected, ranging from as few as 0 ulcers per sector to >100 per sector on the same fish. Although the

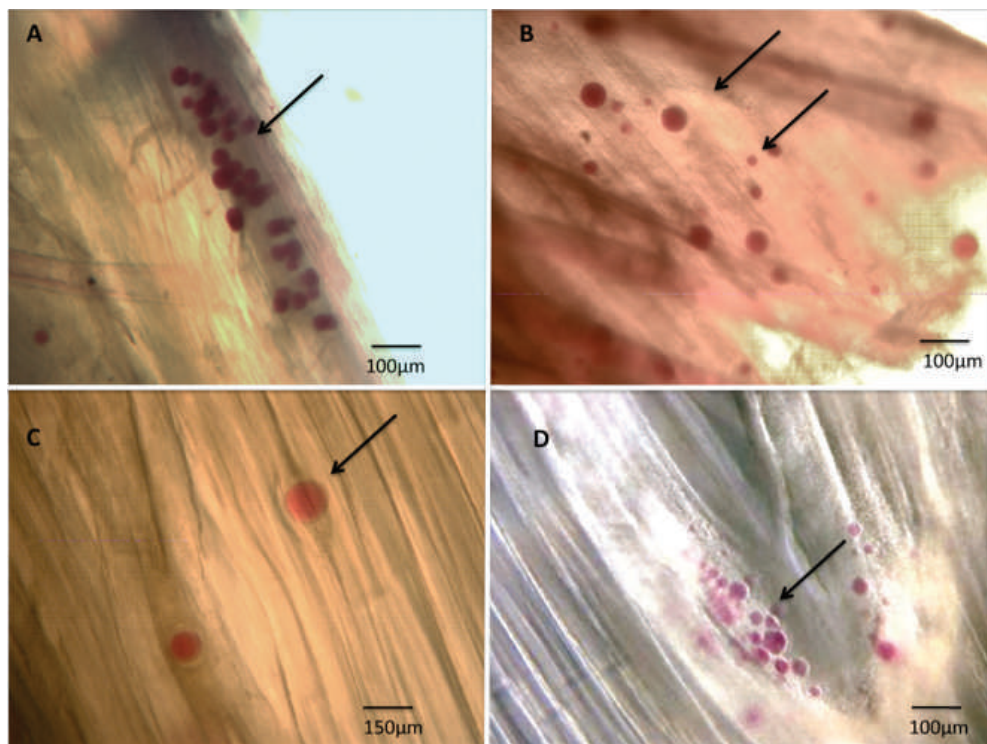


FIGURE 2. Three-dimensional view of *Ichthyophonus* schizonts in situ from different Pacific herring. (A) Clustering of similar size schizonts in the dark muscle (arrow). (B) Random distribution of various size schizonts throughout white muscle (arrows). (C) Low-density of mature schizonts. (D) Clustering of various sized schizonts in white muscle. Schizont diameter: 42- μ m to 117- μ m (stain, PAS following clearing).

ulcers contain numerous viable and infectious *Ichthyophonus* sp. cells (Kocan et al., 2010), they were not predictive of the density of *Ichthyophonus* sp. cells in the underlying musculature (Fig. 3). This lack of correspondence is further supported by the fact that many infected herring do not exhibit external lesions, even when the parasite is present in the underlying musculature. High and low tissue parasite density values presented in Table IV show that schizont density was as low as 0 in some sectors, while other sectors in the same fish had densities $>3,000$ parasites per gram. In addition, we observed that in some fish the schizonts were randomly distributed throughout the white muscle, while in others they occurred in clumps or concentrated in the dark muscle under the skin in the vicinity of the lateral line (Fig. 2). This wide

variability of parasite distribution within the same tissue of a single fish likely accounts for differences in sensitivities among different diagnostic techniques. The age of the infection also influences the parasite burden, as well as distribution of the parasite among organs, thus making the selection of tissue for sampling a critical factor in accurately detecting infected individuals. Rand and Cone (1990) were unable to detect clinically infected fish, e.g., visible lesions, during the first week following experimental exposure of trout, but did detect infected individuals microscopically during the second week. By the sixth week post-exposure, 100% of the fish exhibited visible gross lesions, thus demonstrating that the age of infection influences the probability of detecting this pathogen.

For epidemiologists to accurately evaluate the impact of infection prevalence or intensity of infection on mortality rates, they must have accurate data on the proportion of infected individuals within a population. In light of this requirement, in vitro culture of *Ichthyophonus* sp. appears to be the most sensitive method for detecting the organism in living (or recently dead) individuals and should be considered the diagnostic standard. Histological evaluation, along with histochemistry, is best used to confirm the identity of the parasite and to determine cellular damage and host cell response, but it is not always suitable for accurately screening populations for *Ichthyophonus* sp. prevalence. Techniques such as observation of epidermal ulcers and tissue squash examination are useful for cursory field evaluation during ichthyophoniasis epizootics and associated fish kills where the intensity of infection is typically high but are likely to grossly underestimate true infection prevalence during non-epizootic periods. Likewise, observation of visible punctate or diffuse lesions on internal organs is most useful when the infection is mature but should be verified by the use of other more specific

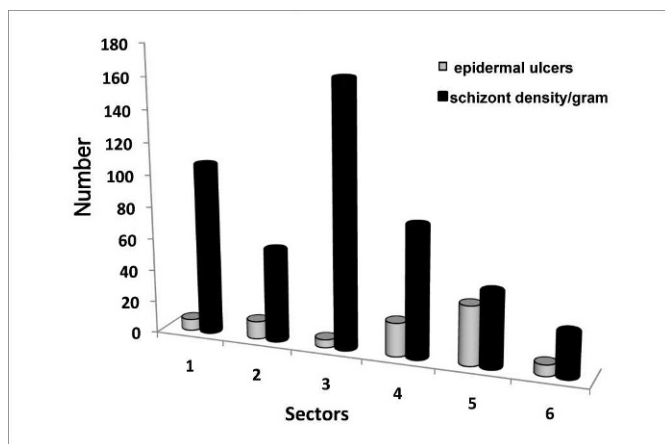


FIGURE 3. Mean number of epidermal ulcers compared with parasite density per gram of skeletal muscle for each of 6 sectors. (Only fish with ulcers used for comparison).

techniques such as in vitro culture and histochemical staining. Molecular techniques such as PCR are useful in revealing the presence of parasite nucleic acid and confirming presumptive diagnoses, but they cannot determine whether live parasites are present.

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