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Analytical and diagnostic performance of a qPCR assay for *Ichthyophonus* spp. compared to the tissue culture 'gold standard'

Vanessa C. Lowe[1,*], Paul K. Hershberger[2], Carolyn S. Friedman[3]

[1]Resource Assessment and Conservation Engineering Division, Alaska Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 7600 Sand Point Way NE, Seattle, WA 98115, USA

[2]Marrowstone Marine Field Station, Western Fisheries Research Center, U.S. Geological Survey, 616 Marrowstone Point Rd., Nordland, WA 98358, USA

[3]School of Aquatic and Fishery Sciences, College of the Environment, University of Washington, 1122 NE Boat St, Seattle, WA 98105, USA

ABSTRACT: Parasites of the genus *Ichthyophonus* infect many fish species and have a non-uniform distribution within host tissues. Due in part to this uneven distribution, the comparative sensitivity and accuracy of using molecular-based detection methods versus culture to estimate parasite prevalence is under debate. We evaluated the analytical and diagnostic performance of an existing qPCR assay in comparison to the 'gold standard' culture method using Pacific herring *Clupea pallasii* with known exposure history. We determined that the assay is suitable for use in this host, and diagnostic specificity was consistently high (>98%) in both heart and liver tissues. Diagnostic sensitivity could not be fully assessed due to low infection rates, but our results suggest that qPCR is not as sensitive as culture under all circumstances. Diagnostic sensitivity of qPCR relative to culture is likely affected by the amount of sample processed. The prevalence values estimated by the 2 methods were not significantly different when sample amounts were equal (heart tissue), but when the assayed sample amounts were unequal (liver tissue), the culture method detected a significantly higher prevalence of the parasite than qPCR. Further, culture of liver also detected significantly more *Ichthyophonus* infections than culture of heart, suggesting that the density and distribution of parasites in tissues also plays a role in assay sensitivity. This sensitivity issue would be most problematic for fish with light infections. Although qPCR does not detect the presence of a live organism, DNA-based pathogen detection methods provide the opportunity for alternate testing strategies when culture is not possible.

KEY WORDS: *Ichthyophonus* · qPCR · Validation · Sensitivity · Specificity · Pacific herring · *Clupea pallasii*

INTRODUCTION

Proper pathogen detection is a first step in accurately estimating disease prevalence, distribution and spread, all of which are fundamental components of epidemiological studies. It is essential to compare new diagnostic methods to established techniques. By comparing results from 2 techniques performed on the same set of samples, one can determine the effectiveness of a new test relative to an existing 'gold standard,' a diagnostic test that is considered the most accurate test for determining the presence of a pathogen and is 'limited to methods that unequivocally classify animals as infected/exposed or uninfected' (OIE 2010, Chapter 1.1.4/5, p. 12; Stojanović et al. 2014). To determine the utility

*Corresponding author: vcannabella@gmail.com

of the new test for the target population, both analytical and diagnostic performance indicators need to be thoroughly evaluated (Purcell et al. 2011). This validation is especially important for molecular-based technologies that do not allow for visual confirmation of pathogens.

Members of the genus *Ichthyophonus* are parasites of economic significance in both wild and cultured fisheries (McVicar 2011). These multinucleated, primitive protists affect numerous fish species with varying disease signs and severity and are present in both marine and freshwater environments (Rahimian & Thulin 1996, McVicar 2011). The genus *Ichthyophonus* may represent an assemblage of related organisms whose phylogeny has yet to be sufficiently resolved; therefore, this parasite will hereinafter be referred to by the genus alone, *Ichthyophonus*. The recommended diagnostic 'gold standard' for *Ichthyophonus* detection in Chinook salmon *Oncorhynchus tshawytscha* is *in vitro* explant culture (Kocan et al. 2011, Hershberger 2012). In addition to explant culture, many other detection methods for *Ichthyophonus* are available, including traditional histology, chromogenic *in situ* hybridization, conventional PCR (cPCR), and quantitative PCR (qPCR) (Kocan et al. 1999, Whipps et al. 2006, White et al. 2013, Conway et al. 2015). It has been suggested, with some debate, that cPCR can be as accurate as culture for detection of this parasite (Hamazaki et al. 2013a,b, but see LaPatra & Kocan 2013). To determine the suitability of cPCR as a field surveillance tool, Hamazaki et al. (2013a) tested samples of wild-caught Chinook salmon by culture and cPCR, and compared the results. Even though cPCR results were promising, the study by Hamazaki et al. (2013a) received criticism, in part due to concern surrounding the true infection status of the fish, as the actual infection status of each fish cannot be known using this experimental design (LaPatra & Kocan 2013) in comparison to a design using known disease-free, and experimentally infected fish groups. Without exposure history, the specificity and sensitivity of a test may not be accurate, and both false negatives and false positives may be misinterpreted.

A validated qPCR assay for *Ichthyophonus* was developed for use on walleye pollock *Gadus chalcogrammus* and was shown to be a more sensitive test for the parasite than cPCR in field tests (White et al. 2013); however, the study did not compare qPCR to culture, an important step for proper assay validation. Although qPCR has inherent limitations common to other molecular-based tests such as the cPCR assay (Whipps et al. 2006, LaPatra & Kocan 2013, White et al. 2013), it may prove to be a useful diagnostic tool for detecting *Ichthyophonus.*

In this study, we evaluated the analytical and diagnostic performance of the qPCR assay for *Ichthyophonus* developed by White et al. (2013) to better understand its usefulness for parasite prevalence estimation relative to the 'gold standard' method for *Ichthyophonus* detection. We evaluated analytical specificity (ASp, the ability to detect DNA target in the presence of the other sample components) and sensitivity (ASe, the smallest amount of target detectable in a sample), as well as diagnostic sensitivity (DSe) and specificity (DSp) by comparing the results of qPCR and culture using Pacific herring *Clupea pallasii* with known exposure histories (OIE 2010). The specific objectives of this study were to (1) estimate the number of *Ichthyophonus* schizonts and DNA copies per dose of live culture inoculum, (2) determine if the qPCR assay is appropriate for use on heart and liver tissues of Pacific herring (selectivity component of ASp for a host species previously untested with qPCR), (3) evaluate the end point cut-off or limit of detection (LOD) of the qPCR assay through practical application of OIE 'bench-level' and 'theoretical' definitions of LOD (reassess ASe), and (4) determine the proportion of positive samples that test positive (DSe) and negative samples that test negative (DSp) by the qPCR assay via cross classifying culture and qPCR results for each treatment and tissue type. This study will provide valuable information regarding the use of this molecular-based assay as an alternative to the existing 'gold standard' for *Ichthyophonus* prevalence estimation.

MATERIALS AND METHODS

To assess the performance of the *Ichthyophonus* qPCR assay relative to culture, we inoculated specific pathogen-free (SPF) Pacific herring with either phosphate-buffered saline (PBS) or an inoculum of live *Ichthyophonus* cultures (100 µl dose aliquots of the inoculum were preserved for further analysis). Heart and liver tissues from control (PBS) and exposed fish were then tested for *Ichthyophonus* presence with both the qPCR assay and culture method. A sampling design was chosen to allow for estimation of DSe and DSp by cross-classification of binary results from each sample and test method. Minimum sample size was determined from a theoretical number table presented by OIE to assess the diagnostic performance of an assay with a minimum DSe and DSp of 90% each. At a 95% confidence level, allowing for 5%

error in the estimate, a minimum of 138 SPF fish would be required to establish a DSp estimate. Similarly, 138 *Ichthyophonus*-infected fish would be needed to establish a DSe estimate.

At the Marrowstone Marine Field Station (MMFS, Nordland, WA, USA), 479 Age-1 SPF Pacific herring were reared from naturally spawned eggs in a controlled pathogen free laboratory environment to ensure no previous exposure to *Ichthyophonus* as described by Gregg et al. (2012). Prior to initiating the experiment, the fish were allowed to acclimate to 9°C water for 5 d. Following the holding period, fish were anesthetized in a bath of buffered seawater with tricaine methanesulfonate (Tricaine-S, Western Chemical) and then inoculated with either PBS or an inoculum of *Ichthyophonus* (described below), each delivered to the fish body cavity via intraperitoneal injection. Fish in the SPF control group (n = 169) were injected with PBS (100 µl $fish^{-1}$) and transferred to one 760 l tank. Because not all fish exposed to *Ichthyophonus* become infected, a minimum of 276 exposed fish would be needed to establish a DSe estimate assuming a 50% infection rate. Fish in the treatment group (n = 310) were exposed to *Ichthyophonus* (100 µl inoculum $fish^{-1}$) and transferred to a second 760 l tank. Control and exposed fish were held at 9°C post-exposure and fed a pellet diet (Bio Vita Starter, Bio-Oregon) to satiation every other day.

Due to logistical constraints, the fish were sampled on 2 sampling dates. Three weeks post-exposure, approximately one-third of the fish from both exposed and control tanks were sampled. The remaining fish from both tanks were sampled 7 wk post-exposure. Dead fish were removed from tanks as they appeared throughout the experiment and were not tested for the parasite. Each fish was euthanized with an overdose of tricaine methanesulfonate, measured, and weighed. Mean ± SD length and weight at time of sampling were 130 ± 13 mm and 26.5 ± 7.5 g for control fish (n = 139), and 131 ± 14 mm and 29.1 ± 8.7 g for exposed fish (n = 298). Fish were then necropsied and the heart was aseptically removed and split equally into 2 samples: one half was placed in Eagle's minimal essential medium buffered to pH 7.8 with Tris, supplemented with fetal bovine serum (5% v/v), penicillin (100 IU ml^{-1}), streptomycin (100 µg ml^{-1}), and gentamycin (100 µg ml^{-1}) for culturing, and the other half was preserved in 100% ethanol for qPCR analysis. Similarly, 2 similar sized pieces of liver were aseptically removed, of which one piece was placed in culture media and the other in 100% ethanol.

Culture samples were incubated at 15°C for 14 d and then examined with an inverted scope under 40× magnification for the presence of *Ichthyophonus*. To detect the presence of *Ichthyophonus* DNA from the ethanol-preserved samples, we followed the methods of White et al. (2013). DNA was extracted from approximately 20 mg of heart or liver tissues; for most of the heart samples, the entire preserved sample was extracted, whereas for all of the liver samples, only a portion of the preserved sample was extracted. DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol, except that DNA was eluted in 100 µl of Buffer AE. Between samples, tools were dipped in 10% bleach, rinsed in distilled water, dipped in ethanol, and flamed. The qPCR reactions of extracted heart tissues were prepared in 25 µl volumes consisting of 2 µl extracted DNA template, *Ichthyophonus* 18S specific primers and probe (400 nM vc7F and vc5R primers, 300 nM 6-FAM-labeled probe ICH27), 15 µg bovine serum albumin (BSA), 12.5 µl 2× SensiMix™ II Probe mastermix (Bioline), and deionized H_2O (White et al. 2013). The qPCR reactions of extracted liver tissues were prepared in 20 µl volumes consisting of 2 µl extracted DNA template, *Ichthyophonus* 18S specific primers and probe (400 nM vc7F and vc5R primers, 300 nM 6-FAM-labeled probe ICH27), 15 µg BSA, 10 µl 2× GoTaq® Probe Master Mix (Promega), and deionized H_2O. Samples were run in duplicate, in a multiplexed qPCR reaction with exogenous internal amplification control (IAC) primers, HEX-labeled probe, and IAC template (single concentration) (Nolan et al. 2006) to test for inhibition relative to the *Ichthyophonus*-free no-template control (NTC). Each 96-well reaction plate comprised 7 serially diluted *Ichthyophonus* standards, an *Ichthyophonus* positive control, and unknowns in duplicate as well as 4 NTCs. The qPCR assay was conducted on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories), using the manufacturer's software. For samples run with SensiMix™ mastermix (hearts), the polymerase was activated for 10 min at 95°C, followed by 40 amplification cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. For samples run with GoTaq® Master Mix (livers), the polymerase was activated for 2 min at 95°C, followed by 40 amplification cycles at 95°C for 15 s and 60°C for 1 min.

The inoculum of live *Ichthyophonus* cultures used in this experiment was generated by pooling *Ichthyophonus* life stages originally isolated from heart explant cultures from wild Pacific herring; *Ichthyophonus* life stages were rinsed and suspended in PBS. Based on microscopic examination,

the inoculum consisted primarily of large (>100 µm) schizonts; however, an unquantifiable number of smaller parasitic stages also existed in the inoculum (<10% of the inoculum); it is unknown if these smaller parasite life stages are infective. To quantify the mean number of schizonts per 100 µl dose, 3 samples were counted and 3 samples were preserved in ethanol for qPCR analysis. To quantify the copies of *Ichthyophonus* DNA per dose, each ethanol-preserved inoculum was digested with 5 times the DNeasy Blood & Tissue extraction kit (Qiagen) manufacturer's standard amount of Buffer ATL (900 µl) and Proteinase K (100 µl) in the original sample tube to make a homogenous solution of 1000 µl and then divided into 5 extractions to prevent overloading a single extraction column. Extracted *Ichthyophonus* DNA concentration was measured using a NanoDrop-2000 spectrophotometer (NanoDrop Technologies). The qPCR reactions of extracted inoculum aliquots were performed following the method used for Pacific herring liver samples described above.

Analytical performance

The selectivity component of ASp refers to the assay's ability to accurately quantify the DNA target in the presence of sample matrix interferents (OIE 2010) and was previously evaluated for the *Ichthyophonus* qPCR assay for application to walleye pollock skeletal muscle or heart tissues (White et al. 2013). To confirm assay selectivity for Pacific herring heart and liver tissues, an *Ichthyophonus* plasmid (IchP) standard curve was constructed following White et al. (2013) resulting in 10-fold serial dilutions of quantified, linearized, *Ichthyophonus* recombinant DNA plasmid in 2 different extracted DNA diluents (SPF Pacific herring heart and liver) as well as the standard diluent, Tris EDTA buffer (TE). Each IchP standard curve in DNA diluents was assayed in triplicate on the same plate paired with the IchP standard curve in TE to determine whether qPCR efficiency (E) and precision were within acceptable ranges, and to compare regression coefficients. Efficiencies between 90 and 110% were considered acceptable and calculated from the slope of the IchP standard curve using the following formula $E = [10^{(-1/slope)}] - 1$ (Stratagene 2004). IchP standard curve precision was evaluated via coefficients of determination (R^2) and values greater than 0.985 were considered acceptable (Stratagene 2004). Regression coefficients were compared following an *F*-variance ratio test (Burns et al. 2005). Similarly, IchP standard curves in TE and extracted heart DNA were assayed with 2 different qPCR reaction mastermixes and associated thermal profiles to determine if a change in mastermix affects the qPCR assay's ability to detect *Ichthyophonus* DNA. Additionally, an IAC (Nolan et al. 2006) was multiplexed with reactions to determine whether PCR amplification was inhibited (increased or absent quantification cycle [Cq] of the IAC). The exclusivity and inclusivity components of Asp, i.e. cross-reactivity with other related genera and the ability to detect species within the genus, respectively, were previously evaluated for the qPCR assay (White et al. 2013) and were not reevaluated for this experiment.

In the laboratory, White et al. (2013) determined that the 'bench-level' ASe of the *Ichthyophonus* qPCR assay is 1 copy reaction^{-1} by the OIE (2010) definition; based on 'theoretical' limits of qPCR detection described by Bustin et al. (2009), the lower limit of detection of a qPCR assay is 3 copies reaction^{-1}. To evaluate ASe from a practical application standpoint, we determined the status (positive, negative) of each unknown sample based on 2 cutoff points following (1) White et al. (2013), where unknown samples were considered positive if the average quantity of replicates was ≥1 copy, determined by following the OIE (2010) definition, and (2) the more conservative Bustin et al. (2009) threshold, where unknown samples were considered positive if the average quantity of replicates was ≥3 copies.

Diagnostic performance

The DSe and DSp were assessed for each tissue type by cross-classifying results from culture and qPCR for exposed fish and control fish separately. For the culture method, presence/absence results were recorded for the heart and liver tissue from each fish. For the qPCR assay, 4 presence/absence results were recorded for each fish, 2 for each tissue type based on a ≥3 copies reaction^{-1} cut-off and a ≥1 copy reaction^{-1} cut-off. Prevalence estimates for the fish were calculated under each method, and differences were determined using a 2 × 2 chi-square.

RESULTS

The mean ± SD count of 3 dose aliquots (100 µl) of live *Ichthyophonus* culture inoculum was 434 ± 77.4 mature schizonts dose^{-1} plus an unquantifiable number of other *Ichthyophonus* life stages. Based on the estimated number of DNA copies dose^{-1} and

the count of schizonts dose^{-1}, each multinucleated *Ichthyophonus* schizont has thousands of copies of the DNA target cell^{-1} (Table 1), and the number of nuclei schizont^{-1} is variable with cell size; therefore copies nucleus^{-1} could not be determined from this study.

Analytical performance

The selectivity component of ASp was evaluated for interference from host material in the sample matrix; the presence of Pacific herring heart- and liver-extracted DNA did not measurably inhibit the assay's ability to detect the *Ichthyophonus* DNA target. For a linear range of 3 to 3 × 10^6 copies, efficiency and precision of IchP standard curves prepared in different extracted DNA diluents were not significantly different from the IchP standard curve prepared in TE (absence of matrix interferents) and were within the acceptable ranges of 90 to 110% efficiency, $R^2 > 0.985$ (Table 2a). Additionally, when the assay was multiplexed with an IAC to test for inhibition, the Cq range of the IAC target for all diluents was within a range of 1.5 Cq per run for IchP standards 3 to 3 × 10^4 copies (Fig. 1). The 2 highest IchP standards, 3 × 10^5 and 3 × 10^6, did have an increased Cq for the IAC, but this increase was seen across all diluents including TE (Fig. 1), and was not likely a result of the presence of host material (see mastermix comparison below). For unknown samples, positive control, and NTCs, the Cq range per run of the IAC target was less than 1.5 Cq (mean = 0.89), suggesting that qPCR reaction inhibition was not a factor for the samples tested.

Two commercially available qPCR mastermix solutions were used in this study; an evaluation of the solutions indicated that the qPCR assay is robust and remained unaffected by mastermix choice in 2 different diluents, i.e. TE and extracted Pacific herring heart DNA. A comparison of assay performance for the detection of *Ichthyophonus* target in IchP standard curves using the 2 different mastermixes and

Table 1. *Ichthyophonus* DNA (ng) extracted per 100 µl dose replicate. The qPCR estimation of *Ichthyophonus* DNA copies per replicate^{-1} and schizont^{-1} based on a mean count of 434 schizonts dose^{-1}

Dose replicate	DNA extracted dose^{-1} (ng)	Copies dose^{-1}	DNA copies schizont^{-1}
A	2770	5.84 × 10^6	1.35 × 10^4
B	2810	6.49 × 10^6	1.50 × 10^4
C	3020	6.11 × 10^6	1.41 × 10^4

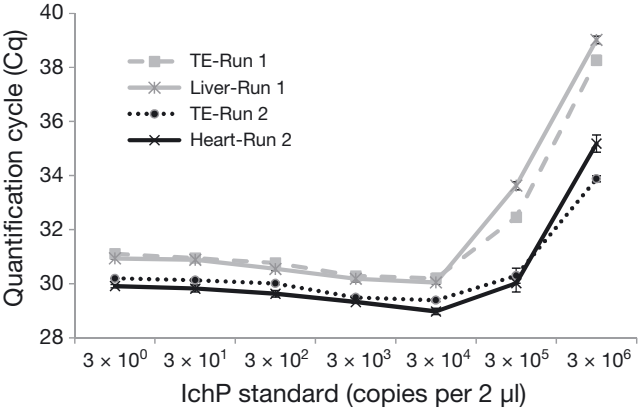

Fig. 1. Average quantification cycle (Cq) of an internal amplification control (IAC) per *Ichthyophonus* plasmid (IchP) standard in 3 diluents: Tris EDTA buffer (TE) and extracted *Clupea pallasii* liver and heart DNA. IchP standard curves assayed in pairs on 2 separate plate runs using Promega GoTaq® mastermix. Error bars indicate ± SE of the mean (n = 3)

Table 2. *Ichthyophonus* qPCR assay efficiency, precision, and quantification cycle (Cq) range of *Ichthyophonus* plasmid (IchP) standard curves prepared in different diluents for (a) selectivity component of analytical specificity (ASp) for *Clupea pallasii* heart and liver tissues; and (b) mastermix and associated thermal profile comparisons (SensiMix™ Probe II vs. GoTaq® Probe). The linear range for all IchP standard curves is 3 to 3 × 10^6 copies; rxn: reaction

Comparison	Diluent (ng DNA rxn^{-1})	Mastermix	Cq range (%)	Efficiency (R^2)	Precision	p
(a) Different diluents (effect of matrix interferents from host tissues)	Tris EDTA Buffer (0)	GoTaq	18.09–38.17	97.7	0.998	0.396
	Extracted liver DNA (2600)	GoTaq	17.76–38.38	96.3	0.999	
	Tris EDTA Buffer (0)	GoTaq	18.26–39.05	94.3	0.999	0.180
	Extracted heart DNA (726)	GoTaq	17.73–38.30	96.1	0.999	
(b) Mastermixes & associated thermal profiles	Tris EDTA Buffer (0)	Sensimix	19.09–39.58	97.6	0.999	0.962
	Tris EDTA Buffer (0)	GoTaq	18.01–38.32	97.6	0.999	
	Extracted heart DNA (726)	Sensimix	18.31–39.01	95.9	0.996	0.879
	Extracted heart DNA (726)	GoTaq	17.73–38.30	96.1	0.999	

associated thermal profiles revealed that there was no significant difference (Table 2b) between the performance of the mastermix used for assay design (SensiMix™ Probe II; White et al. 2013) and an alternative mastermix (GoTaq® Probe). For a linear range of 3 to 3×10^6 copies, efficiency and precision of IchP standard curves assayed in different mastermixes were within the acceptable ranges of 90 to 110% and $R^2 > 0.985$, respectively (Table 2b). Although the ability to accurately quantify the *Ichthyophonus* DNA target was unaffected by the change in mastermix, the multiplexed IAC did perform differently in reactions with high concentrations of recombinant *Ichthyophonus* DNA; 3×10^5 and 3×10^6 IchP standards had increased Cq with the GoTaq® Probe Master Mix regardless of diluent (Fig. 2).

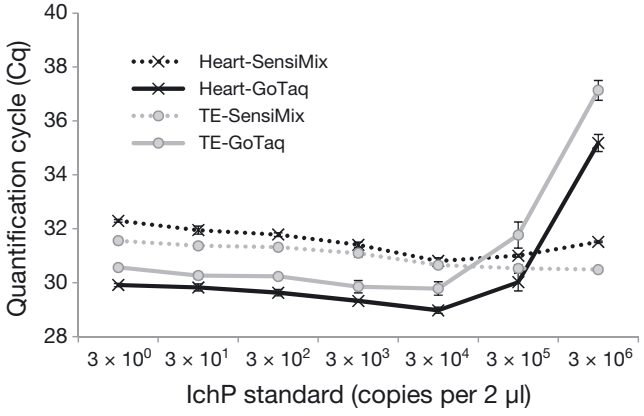

Fig. 2. Average quantification cycle (Cq) of an internal amplification control (IAC) per *Ichthyophonus* plasmid (IchP) standard in Tris EDTA buffer (TE) and extracted *Clupea pallasii* heart DNA. Using 2 different mastermixes, SensiMix II™ Probe and GoTaq® Probe, the IchP standard curve was assayed on 2 separate plate runs using 2 different thermal profiles specific to each mastermix. Error bars indicate ±SE of the mean (n = 3)

Diagnostic performance

Results from the 2 tests for *Ichthyophonus* were obtained for 437 Pacific herring (Table 3). The DSp using qPCR on control tank fish was 100% (n = 139) regardless of tissue type or qPCR LOD. The qPCR DSe varied with LOD and tissue type tested (Table 3). *Ichthyophonus* prevalences estimated by culture and qPCR using heart tissue were similar, while prevalence estimated by culture of liver tissue was significantly higher than that estimated by qPCR of liver tissue (Table 3).

DISCUSSION

When applying a validated qPCR assay for pathogen detection to a new sample type, host species, or tissue, it is important to determine that the assay performs equally as well as it did under validation conditions. While our experimental results did not support the notion that qPCR can be used as a replacement for the 'gold standard,' and the sample size of infected fish was too low to complete 1 goal of the study, we did accomplish 3 of the 4 objectives to eval-

Table 3. Diagnostic sensitivity (DSe) and specificity (DSp) of the *Ichthyophonus* qPCR test on experimentally inoculated *Ichthyophonus*-exposed (ICH) and control (PBS) *Clupea pallasii*. Cross-classified culture (C) and qPCR (Q) results are presented individually for fish heart and liver tissues at 2 different qPCR limits of detection (LOD) for parasite DNA in fish tissue with associated DSe and DSp. Prevalence estimates are listed for each detection method with chi-squared p-values from comparing each pair. Prevalence values are percentages (95% binomial proportion CI). na: not applicable

Inoculum/ Tank	qPCR Tissue	LOD	Number of samples n	C+Q+	C+Q−	C−Q+	C−Q−	Infection prevalence (%) Culture	qPCR	p	Diagnostic performance DSe	DSp
PBS	Heart	≥3	139	0	0	0	139	0	0	–	na	100
		≥1		0	0	0	139	0	0	–	na	100
	Liver	≥3	139	0	0	0	139	0	0	–	na	100
		≥1		0	0	0	139	0	0	–	na	100
ICH	Heart	≥3	298	17	2	3	276	6.4 (4.1–9.8)	6.7 (4.4–10.1)	0.868	89.5 (69–97)	98.9 (97–100)
		≥1		18	1	4	275	6.4 (4.1–9.8)	7.4 (4.9–11.0)	0.627	94.7 (75–99)	98.6 (96–99)
	Liver	≥3	296	21	25	1	249	15.5 (11.9–20.1)	7.4 (5.0–11.0)	0.002	45.7 (32–60)	99.6 (98–100)
		≥1		23	23	4	246	15.5 (11.9–20.1)	9.1 (6.3–12.9)	0.018	50 (36–64)	98.4 (96–99)

uate the analytical and diagnostic performance of the qPCR assay for *Ichthyophonus*. The qPCR assay evaluated in this study is a specific, robust assay capable of detecting *Ichthyophonus* DNA in Pacific herring heart and liver tissues, and in some circumstances can provide results comparable to that of the culture method.

To address our first study objective, we used the qPCR assay to estimate the number of *Ichthyophonus* DNA copies dose^{-1} of inoculum and compared that to the estimated number of schizonts dose^{-1} and found that there are thousands of copies of the 18S rDNA gene in each schizont and millions of copies per 100 µl dose (Table 1). Because the number of schizonts dose^{-1} is proportionally related to infection intensity when live cultures are used to experimentally expose SPF fish (Kocan & LaPatra 2016), qPCR may be a useful tool for standardizing doses across experiments considering that schizont size is highly variable. The finding of a high copy number schizont^{-1} also highlights the sensitivity of the qPCR assay, considering it can detect the presence of only a few copies of the target DNA, equivalent to a small fraction of an individual *Ichthyophonus* cell. However, this result is not surprising considering *Ichthyophonus* schizonts are multinucleated and the target region, the 18S rRNA gene, is repetitive in nature (Meyer et al. 2010). This multicopy gene is not well characterized, and the number of gene copies varies among taxa. Within the kingdom Fungi, for example, 18S rRNA gene repeats can range from tens to hundreds (Black et al. 2013); it is not known how many repeats of 18S exist for *Ichthyophonus*.

By comparing qPCR performance on a standard curve of target DNA in different diluents, we addressed the second objective of the study and verified the selectivity component of ASp for a fish host species previously untested with qPCR. Assay performance was not affected by the presence of host extracted DNA in the sample matrix, and test results indicate that the *Ichthyophonus* qPCR assay is appropriate for use on both Pacific herring heart and liver tissues (Table 2a). This finding is important because certain tissues and substances can have inhibitory factors that may affect the molecular reactions (Rådström et al. 2004), resulting in false negative results (low DSe); that is, the target DNA is present but does not amplify due to the presence of inhibitors.

To look for inhibitory effects for individual unknown samples, we multiplexed the assay with an IAC. Amplification of the second DNA target (IAC) present in each sample at the same concentration was not affected, so inhibition did not appear to be a factor for these individual Pacific herring samples. For the IchP standard curve, however, a consistent and progressive increase in the IAC Cq was apparent for the highest 2 IchP standards regardless of diluent (Fig. 2), but only when using a new mastermix, GoTaq® Probe. This problem was only apparent for the IAC, as amplification of the IchP standard curve (*Ichthyophonus* DNA) did not appear to be affected (Table 2b). This consistent anomaly may be a result of dNTP limitation due to the overabundance of *Ichthyophonus* DNA target in the highest IchP standards. This issue could likely be corrected for the GoTaq® Probe mastermix with further optimization of the assay, for example, primer limitation of the more abundant target or an increase in dNTP concentration. We did not pursue further assay optimization because the IAC amplification in individual samples was not affected, nor the IchP standard curve (Table 2).

The end point cut-off or lower LOD of an assay is frequently assessed using a bench-level approach by determining the target concentration by which a minimum of 50% of replicates yield positive results (OIE 2010) or a theoretical minimum level based on statistical theory (Bustin et al. 2009). For the *Ichthyophonus* qPCR assay, White et al. (2013) determined that the bench-level LOD is ≥1 copy reaction^{-1}, and ≥3 copies reaction^{-1} based on theoretical limits. When the assay is applied to samples from a host population, however, false positives may occur if the LOD is set too low as a result of spurious or non-specific amplification that can occur late in reaction cycling. Alternatively, if the LOD is set too high, false negatives may result, ultimately reducing the overall sensitivity of the assay. Therefore, when possible, it is valuable to test the assay on fish with known health history and select the LOD based on evidence from testing that host population. To address objective 3, we reassessed ASe from this practical standpoint and determined that the 2 aforementioned LODs are both functional in practice, but the 1 copy reaction^{-1} cut-off yields more positive results and a higher prevalence estimate for both tissue types without additional false positives for this species held in captivity (Table 3). Further, no false positives were detected in control fish for either LOD or tissue type tested (Table 3). These findings support the use of the qPCR assay in future captive studies on Pacific herring, but use of the assay should be reassessed if applying it to environmental samples (e.g. water, sediment, or plankton samples) or used as a preliminary screening test in combination with another confirmatory method. Environmental samples may present more potential for nonspecific amplification from unknown

sources such as microorganisms not found in fish that have yet to be identified.

Diagnostic performance of the *Ichthyophonus* qPCR assay, the final objective, could not be fully evaluated with high confidence due to the limited number of *Ichthyophonus*-exposed fish that developed infections. To reach our error and confidence level goals for DSe following the OIE (2010) guidelines, we required at least half of the exposed fish to acquire the infection. Only 18.5% of the exposed fish acquired the infection based on the least conservative estimate of *Ichthyophonus* prevalence, calculated by assuming a fish was infected if any detection method yielded a positive result. This low infection rate is far below what we anticipated, possibly because of insufficient dose concentration, sub-optimal tank temperature, suboptimal (i.e. preponderance of non-infectious) parasite stages in the inoculum batch, or other unknown variables.

Although the infected fish sample size was too low to fully assess DSe for the qPCR assay in comparison to culture, useful DSe values and information were collected by comparing the results of qPCR and culture for the 2 tissue types assayed (Table 3). By testing fish hearts alone, more fish were identified as *Ichthyophonus*-infected using qPCR than culture, even though the DSe relative to culture was under 95% (Table 3). However, when the same diagnostic tests were applied to the fish liver, qPCR missed about half of the infected fish compared to culture, resulting in a significant underestimate of prevalence (Table 3). The qPCR assay performed similarly for both heart and liver tissues during the analytical performance testing phase (Table 2), so we hypothesize that there are potentially 2 other factors contributing to the prevalence discrepancy between tissue types, namely (1) disease progression in the laboratory-inoculated fish and (2) the amount of sample assayed. For many fish hosts of *Ichthyophonus*, infections are acquired through feeding on infected fish tissue, but for Pacific herring, the natural route of parasite transmission is unknown (Gregg et al. 2012). Therefore, to establish infections in SPF Pacific herring it is common practice to inject live *Ichthyophonus* schizonts into the body cavity of the fish. Because injections were in close proximity to the liver, with potential for direct injection to the organ, perhaps infections presented earlier and/or more often in the liver than in the heart. This could explain why neither culture nor qPCR detected more infected fish when the heart tissue was tested (compared to testing liver), even though this organ is typically targeted for prevalence estimates in wild populations (Kocan & Hershberger 2006, Hershberger et al. 2010).

The discrepancy in assay performance between the 2 tissue types may also have been affected by individual sample size. *Ichthyophonus* has a non-uniform distribution within tissues, which complicates sensitivity tests and is frequently offered as an explanation for a lack of concordance among diagnostic tests (Whipps et al. 2006, Kocan et al. 2011, White et al. 2013). If only a small portion of the sample can be examined (i.e. a thin section in histology or a tiny piece of tissue extracted for molecular tests), the test will only be accurate if the parasite happens to be present in the sample. The culture method can accommodate a large piece of tissue for examination without replicate samples and added cost. In part, this is why the culture method yields the most accurate estimate of prevalence in fish populations, earning the label as the 'gold standard' (Kocan et al. 2011). This concept is illustrated by the results of this study. The age-1 Pacific herring used in this study have relatively small hearts, which were split in half for the 2 diagnostic methods. In most cases (83.3%), the entire heart sample was extracted, yielding a homogenous DNA sample representative of half the heart organ for qPCR testing, comparable to performing culture on the other half; the resulting prevalence estimates from these 2 methods were comparable (Table 3). In contrast, the liver samples collected for each diagnostic method were not necessarily the same size due to the relatively larger size of this organ and availability of more tissue. For all liver samples, only a portion of the preserved liver sample was extracted due to limitations of the extraction column (Qiagen 2006). In this example, qPCR only assayed a fraction of the liver tissue that was assessed by the culture method; accordingly, the sensitivity of qPCR was greatly reduced in comparison to culture resulting in an underestimate of prevalence (Table 3). Consequently, if culture is not feasible for an experiment or field study, a focus on sample collection and extraction methodology to increase sample mass would likely maximize accuracy when using molecular tests. For example, to process a larger piece of tissue without the added cost of extracting and running replicates, one could lyse the entire sample (additional cost of lysis buffer and Proteinase K to maintain ratio of tissue to lysing solutions) and then continue the extraction with a subsample of the homogenous lysed mixture. For this methodology, qPCR would be the preferable molecular test to use due to its increased ASe compared to conventional PCR (White et al. 2013), as there is potential for dilution of the target. Perhaps such an approach could increase the DSe of qPCR relative to culture for estimating prevalence in wild populations. A similar approach has proved effective in detecting po-

tato pathogen DNA from relatively large soil samples (Brierley et al. 2009).

The DSp estimate for qPCR generated from both the control tank and exposed tank were consistently high (Table 3), over 98% (95% binomial proportion CI, 95.9–100.0). For fish from the control tank, DSp was 100% for both culture and qPCR (Table 3), as none of the control fish yielded false positive results. This provides us a great deal of confidence in the ASp of this assay, which makes it a good candidate assay to evaluate for other types of applications, such as environmental sample testing, multi-pathogen screening platforms, and testing of archived samples.

Other applications for the qPCR assay may become increasingly important because *Ichthyophonus* is a parasite that affects the health of many commercially important wild fish hosts, including multiple Pacific salmon species and Pacific herring (Hershberger et al. 2002, Kocan et al. 2006, McVicar 2011, Vollenweider et al. 2011). For planktivorous hosts of *Ichthyophonus*, natural sources of infection are unknown (Gregg et al. 2012), and an infective stage of the parasite can live free from the host in seawater for many months (Hershberger et al. 2008). Therefore, the ability to screen environmental DNA (eDNA) samples for *Ichthyophonus* with qPCR could be useful to further our understanding of transmission and non-fish sources of infection. For wild, economically valuable host species that are susceptible to many different pathogens including *Ichthyophonus*, future research needs to address multiple stressors in combination with changing climate conditions to assess health risks. By combining multiple qPCR assays into a high-throughput microfluids platform capable of assessing the presence and load of multiple pathogens at once, researchers can work on broad-scale disease monitoring studies. This objective is being addressed by the Strategic BC Salmon Health Initiative based in Canada, for diseases and pathogens that affect Pacific salmon including *Ichthyophonus,* to better understand ecological and evolutionary consequences of cumulative stressors in wild salmon populations (Miller et al. 2014). Finally, another benefit of molecular-based assays is the ability to test archived host samples to assess historical presence, distribution and prevalence of parasites such as *Ichthyophonus* that could not be obtained through culture techniques. This approach was used for identifying a densovirus responsible for recent mass mortality events that decimated sea-stars on the northeast Pacific Coast. Viral DNA from the causative agent was identified in museum specimens using qPCR, revealing that the seemingly novel pathogen had actually been present in the system for over 70 yr (Hewson et al. 2014).

In conclusion, results from this experiment support that the qPCR assay for *Ichthyophonus* is a robust assay, suitable for application to multiple Pacific herring tissues with high specificity. While the experimental results did not demonstrate with statistical confidence that the qPCR assay is as sensitive as culture for the detection of *Ichthyophonus* in wild populations using currently described methods, we surmise that comparable prevalence estimates could be achieved with an improved extraction method that can accommodate a larger piece of tissue, equivalent to that used in the culture method. Culture is currently the most sensitive detection method for *Ichthyophonus* and provides a visual confirmation of live parasite. However, it can be problematic in some field or environmental settings where significant microbial contamination may be present, and is not stable for long-term storage. A paired sample collected for qPCR analysis would be valuable as a stable backup sample in the event of culture sample loss or contamination. Until extraction methods improve, prevalence estimates from this method may yield an underestimate of *Ichthyophonus* prevalence, especially for populations with light infections (Whipps et al. 2006, White et al. 2013). Reduced sensitivity is an important factor to take into account if this assay is considered for use as part of a molecular-based platform to screen fish samples for multiple pathogens. However, the ability to detect *Ichthyophonus* with such a tool would provide valuable information towards a better understanding of interactions and cumulative effects of multiple pathogens on affected hosts.

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