

Comparison of quantitative PCR and ELISA for detection and quantification of *Flavobacterium psychrophilum* in salmonid broodstock

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ABSTRACT: A quantitative PCR (qPCR) assay was developed for *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease. The assay was targeted to *fp1493* as it encodes a putative outer membrane protein (FP1493) that is reactive to the monoclonal antibody (MAb FL43) used in a standardized *F. psychrophilum* capture enzyme-linked immunosorbent assay (ELISA). The qPCR was specific to *F. psychrophilum* and was able to detect between 8 and 809 000 copies of *fp1493*. To determine if antigen level in the tissue was indicative of bacterial concentration, kidney samples from 108 steelhead *Oncorhynchus mykiss* and coho salmon *O. kisutch* female broodstock were screened by ELISA and qPCR. There was no correlation between ELISA optical density (OD) values and the number of *F. psychrophilum* cells g⁻¹ of kidney tissue as estimated by qPCR ($r_s = 0.42$; $p > 0.05$). The median number of *F. psychrophilum* cells in steelhead samples was 6.11×10^3 cells g⁻¹ of tissue. For coho salmon samples, the median number of cells was 3.95×10^3 cells g⁻¹ of tissue. Agreement between the 2 assays was less than 50%. As *fp1493* is a single-copy gene and differential expression of FP1493 has been reported, we hypothesize that the discrepancy between the 2 assays is due to increased expression of FP1493 in the *in vivo* environment. Therefore, ELISA OD values most likely provide an indication of differential protein expression, while the qPCR assay estimates bacterial load in tissue.

KEY WORDS: *Flavobacterium psychrophilum* · Quantitative PCR · Kidney tissue · ELISA · FP1493

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INTRODUCTION

The causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), *Flavobacterium psychrophilum*, is a pathogen of concern in salmonid aquaculture because disease outbreaks can result in significant mortality in rainbow trout, steelhead (both *Oncorhynchus mykiss*), and coho salmon *O. kisutch* populations. As *F. psychrophilum* can be re-isolated from reproductive fluids, unfertilized eggs, and eyed eggs (Brown et al. 1997, Madsen et al. 2005, Kumagai & Nawata 2011), verti-

cal transmission is likely. We have hypothesized that the incidence of BCWD outbreaks at hatcheries would be reduced if broodstock infection levels were monitored and eggs or progeny from highly infected broodstock were culled. For this strategy to be successful, however, reliable and accurate diagnostic assays are needed to quantify infection levels in tissue samples and/or ovarian fluid. Although several assays have been recently validated for screening broodstock (Long et al. 2012), there are currently only 2 assays that can be used to quantify *F. psychrophilum* or provide an indication of infection

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severity: quantitative PCR (qPCR) and a capture enzyme-linked immunosorbent assay (ELISA) (Lindstrom et al. 2009).

Quantitative PCR (qPCR) has become a valuable diagnostic assay for estimating pathogen load in samples. Published assays for fish pathogens include those for *Renibacterium salmoninarum* (Powell et al. 2005, Chase et al. 2006), *Francisella noatunesis* subsp. *orientalis* (Soto et al. 2010), *Henneguya ictaluri* (Griffin et al. 2008), *Aeromonas salmonicida* (Balcázar et al. 2007), *Flavobacterium columnare* (Panangala et al. 2007), *Nucleospora salmonis* (Foltz et al. 2009), viral hemorrhagic septicemia virus (VHSV) (Matejusova et al. 2008, Garver et al. 2011), and *Myxobolus cerebralis* (Cavender et al. 2004). Although qPCR assays for *F. psychrophilum* have been reported (Orioux et al. 2011, Marancik & Wiens 2013, Strepparava et al. 2014), there have been no studies evaluating the relationship between bacterial concentration and antigen concentration.

The capture ELISA developed by Lindstrom et al. (2009) uses a monoclonal antibody (MAb FL43) specific to a putative outer membrane protein (Lindstrom et al. 2009, Gliniewicz et al. 2012) identified as FP1493 (Gene ID: 5300029) (Gliniewicz et al. 2012), a protein of unknown function. The gene encoding FP1493, *fp1493*, is a single-copy gene 645 nt long, and is present in both the *F. psychrophilum* JIP02/86 (ATCC 49511) (GenBank no. AM398681) (Duchaud et al. 2007) genome and the *F. psychrophilum* CSF259-93 genome (GenBank no. CP007627) (Wiens et al. 2014). Based on previous research (LaFrentz et al. 2009, Gliniewicz et al. 2012, Long et al. 2012), we have hypothesized that FP1493 is a component of the *F. psychrophilum* iron uptake system, and may aid in establishment of infection in the iron-limited host environment. For these reasons, *fp1493* was selected as the gene target for a qPCR assay.

In the current study, we developed a sensitive and specific qPCR assay for *F. psychrophilum* using primers targeting *fp1493*. We then evaluated agreement between the capture ELISA and the qPCR for kidney tissue samples from steelhead and coho salmon broodstock at 2 hatcheries in the Pacific Northwest.

MATERIALS AND METHODS

Sample collection

Kidney samples were collected at time of spawning from 60 female steelhead returning to an inland

hatchery in Oregon (spring 2011; 6 collection events) and 60 female coho salmon (fall 2010; 1 collection event) returning to a coastal facility in Washington. Approximately 1 g of head kidney was aseptically collected from each fish and transported on ice to the University of Idaho within 24 h of collection. Tissue was divided into 0.5 g subsamples and stored at -80°C until needed.

Bacteriological culture

To verify the specificity of the qPCR primers, 7 common fish pathogens and *Flavobacterium* spp. were cultured. *Aeromonas hydrophila*, *A. salmonicida*, and *Yersinia ruckeri* were cultured in tryptic soy broth (TSB; Becton Dickinson) at 22°C . *F. psychrophilum* (strain CSF259-93), *F. saccharophilum* ATCC 49530, *F. johnsoniae* ATCC 17061, *F. branchiophilum*, and *F. columnare* ATCC 23463 were cultured in tryptone yeast extract salts broth (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulfate, pH 7.2) (Holt et al. 1993) at 17°C .

DNA extraction

The DNeasy® Blood & Tissue Kit (Qiagen) was used to extract DNA from kidney samples and bacterial cultures. Bacterial cultures were extracted following the manufacturer's instructions for extraction from Gram-negative bacteria. To extract DNA from kidney, frozen samples were thawed and 25 mg of tissue was minced into small pieces prior to addition of tissue lysis buffer and Proteinase K. Samples were incubated at 56°C for 16 h with periodic vortexing. Extractions were carried out according to manufacturer's protocols for purification of total DNA from animal tissues. Samples were eluted in 150 μl of elution buffer and stored at -20°C . A Synergy HT Multi-Mode Microplate reader with Gen 5.5™ software (BioTek US) was used to quantify DNA and check purity. Kidney tissue DNA was diluted 1:10 in sterile, nuclease-free water prior to use to reduce the effect of inhibitors.

Tissue ELISA

Antigen levels in kidney tissue were estimated by the capture ELISA following the protocol described by Long et al. (2012).

Primer design

A BLAST search of the *fp1493* sequence indicated that the first 180 nt are unique to *F. psychrophilum* and the rest of the gene is similar to a *F. johnsoniae* hypothetical lipoprotein (Gene ID: 5091303). *F. johnsoniae* is ubiquitous in soil and fresh water, and has been identified as an opportunistic fish pathogen (Soltani et al. 1994, Flemming et al. 2007). For this reason, primers were designed for the region of *fp1493* unique to *F. psychrophilum* using Primer Express 3.0 (Applied Biosystems, Life Technologies) (Table 1).

Primer sensitivity and specificity

To estimate the analytic sensitivity of the PCR assay, genomic *F. psychrophilum* DNA (gDNA) was diluted to 0.5 ng μl^{-1} (2.5 ng total) in sterile, nuclease-free water and then serially diluted (10-fold) to a final concentration of 5.0 fg μl^{-1} (2.5×10^{-5} ng total). Each dilution was run in triplicate and the standard curve was run on 4 separate occasions ($n = 12$). Efficiency and linearity of the curve were calculated for each run.

To determine analytic sensitivity of the PCR assay for detecting gDNA spiked into kidney tissue, DNA was extracted from kidney tissue of an unrelated fish species (burbot *Lota lota maculosa*). As burbot are refractory to *F. psychrophilum* (Polinski et al. 2010), kidney tissue from these fish was used in this study to eliminate the risk of background *F. psychrophilum* contamination as seen in kidney sampled from putatively naïve rainbow trout. Purified kidney DNA (kDNA) was diluted 1:10 in sterile, nuclease-free water and tested by qPCR. If there was no amplification, it was then used as the diluent for a standard curve. To do so, kDNA was diluted 1:10 in sterile, nuclease-free water and gDNA was then serially diluted from 0.5 ng μl^{-1} to 5.0 fg μl^{-1} in diluted kDNA. Reactions were run in triplicate on 3 separate occasions ($n = 9$). For each bacterial species tested, purified genomic DNA was diluted to 0.5 ng μl^{-1} in sterile, nuclease-free water and then used. All samples were run in triplicate.

Table 1. Primer sequences, location, and expected product size for the qPCR assay designed for *fp1493* from *Flavobacterium psychrophilum*

Name	Sequence (5'–3')	Location	Product size
FP1493_F	TTC GTG TCT TCT TGT AGT AAA GAC	46–69	110 bp
FP1493_R	ACT TGT GGT GCA TTA AGG TTT GAA	132–155	

Quantitative PCR

Reactions were carried out on an ABI 7500 Fast thermal cycler using the standard option (Life Technologies). An individual reaction was comprised of 12.5 μl of iTaq™ Fast SYBR® Green Supermix with ROX (Bio-Rad Laboratories) mixed with 0.625 μl each of forward and reverse primer (final concentration 0.25 μM), 6.25 μl of sterile, nuclease-free water, and 5 μl of DNA. The *F. psychrophilum* gDNA standard curve was made fresh every day using sterile, nuclease-free water as the diluent. The standard curve was included on every plate along with a no-template control (NTC). Kidney sample DNA was diluted 1:10 in nuclease-free water immediately prior to qPCR. All samples, as well as the standard curve and NTC, were run in triplicate. Reactions were done in MicroAmp® Fast 96-Well Reaction Plates (Applied Biosystems, Life Technologies). Parameters of the reaction were as follows: initial denaturation was carried out at 95°C for 2 min followed by 40 cycles in which each cycle had a denaturation step at 95°C for 15 s and an annealing/extension step at 55°C for 1 min. Melt curve analysis was done for every run, spanning 60 to 95°C with a temperature increase of 1°C min^{-1} . Samples with a mean quantification cycle (Cq) value ≥ 37 were classified as negative because this is the theoretical limit of detection based on the results of gDNA spiked into burbot kDNA (Bustin et al. 2009).

Data analysis

Individual Cq values from each standard curve run were used to calculate mean Cq values and standard deviation (SD) for the genomic DNA diluted in either purified burbot kidney ($n = 9$) or water ($n = 12$). Linear regression was carried out for each independent standard curve for both spiked kDNA and water using the \log_{10} -transformed total DNA concentration (ng) and Cq values to calculate efficiency and linearity. For inter-run (between assay) repeatability, the coefficient of variation (CV) was calculated using mean Cq and SD for all replicates from all runs. For intra-run (within assay) repeatability, CV was calculated using replicates from that run. To calculate the number of *F. psychrophilum* cells g^{-1} of tissue in individual samples, the dilution factor and weight of extracted sample were

accounted for in the calculation. Both ELISA optical density (OD) values and estimated number of cells g^{-1} of tissue were \log_{10} transformed and Spearman's rank correlation coefficient was determined. GraphPad® Prism v 5.03 (GraphPad) was used for all statistical analyses.

RESULTS

Primer efficiency and sensitivity

Mean Cq values and SD for the 2 different diluents are listed in Table 2 along with the inter-run CV values. The efficiency for the individual standard curves for gDNA diluted from 0.5 ng μl^{-1} to 5.0 fg μl^{-1} in water averaged 91% (range 90 to 92%). The R^2 value for each run was 0.999. Intra-run variability was very low with CV values ranging from 0.17 to 2.52%. When gDNA was diluted in kDNA, these curves also had a high degree of linearity but efficiency of the reaction decreased. Efficiency of these runs were 84% ($R^2 = 0.996$), 79% ($R^2 = 0.996$), and 79% ($R^2 = 0.993$) (mean efficiency 81%). Intra-run variability for these samples was very similar to the low values that were calculated for the gDNA diluted in water (0.31% to 2.33%).

Regardless of diluent, we were able to consistently detect between 0.5 ng μl^{-1} (2.5 ng) and 5.0 fg μl^{-1} (2.5×10^{-5} ng) (Table 2). The size of the *Flavobacterium psychrophilum* genome is 2.86 Mb (Duchaud et al. 2007), making these concentrations equivalent to 8 to 809 000 copies of the gene. As *fp1493* is a single copy gene, this indicates that we were able to quantify as few as 8 cells per reaction under idealized conditions. When concentrations above 0.5 ng μl^{-1} were used in the reaction, overabundance of template resulted in linear PCR reactions (data not shown).

Primer specificity

Primers developed for this assay were specific for our purposes because amplification was not ob-

served for non-*F. psychrophilum* species. The melting temperature of the negative control was 65.9°C, while the melting temperature of the correct *F. psychrophilum* product was typically between 76.3 and 77°C.

Quantitative PCR results

In all, 108 steelhead and coho salmon samples were examined with the qPCR assay. The coefficient of variation was low for each sample (<5%), indicating that intra-run variability was low for this type of samples. The estimated median number of cells g^{-1} of tissue for each species is given in Table 3. Overall, a large number of samples were positive although the number above the quantifiable limit (QL) was small. Of the 53 samples analyzed from steelhead spawned in 2011, 74% (39/53) were positive by qPCR. Of those, 46% (18/39) were above the QL. Of the 55 coho salmon samples analyzed, 60% (33/55) were positive by qPCR although only 30% (10/33) were above the QL.

Table 2. Mean quantification cycle (Cq) values \pm standard deviation (SD), inter-run variability (CV), and difference in mean Cq for genomic DNA diluted in either water (n = 12) or purified burbot *Lota lota maculosa* kidney DNA (n = 9)

Total DNA (ng)	Water		Kidney		Difference in Cq
	Mean Cq \pm SD	CV (%)	Mean Cq \pm SD	CV (%)	
2.5	16.84 \pm 0.17	1.00	17.42 \pm 0.35	2.02	0.58
0.25	20.20 \pm 0.25	1.23	21.66 \pm 0.40	1.84	1.46
0.025	23.86 \pm 0.25	1.04	25.33 \pm 0.60	2.35	1.46
0.0025	27.62 \pm 0.31	1.13	29.52 \pm 0.62	2.10	1.90
0.00025	31.30 \pm 0.29	0.93	33.67 \pm 0.81	2.39	2.37
2.5×10^{-5}	34.40 \pm 0.47	1.36	36.55 \pm 0.74	2.01	2.15

Table 3. Results of steelhead *Oncorhynchus mykiss* and coho salmon *O. kisutch* kidney samples analyzed by qPCR and ELISA for the presence of *Flavobacterium psychrophilum*. The number of positive samples that had results above the positive threshold for either assay is reported as well as the median value and the range for these samples. Median values are reported to reduce the influence of outliers

Species	ELISA OD values		qPCR (bacterial cells g^{-1} tissue)	
	No. of positive samples	Median (range)	No. of positive samples	Median (range)
Steelhead	11	0.123 (0.104–0.149)	18	6110 (3650–80600)
Coho salmon	55	0.105 (0.09–0.133)	10	3950 (3030–8390)

ELISA results

A total of 53 steelhead kidney samples were tested for *F. psychrophilum* by ELISA and 21% (11/53) were positive. All of the coho salmon kidney samples (n = 55) were positive by ELISA. The median ELISA OD value for each species is given in Table 3.

Comparison between ELISA and qPCR

There were 5 kidney samples from steelhead and 10 from coho salmon that were both positive by ELISA and the estimated number of cells g^{-1} of tissue was above the QL. Results for the 2 species were combined for analysis. There was no correlation (Spearman's rank: $r_s = 0.42$; $p > 0.05$) between ELISA OD values and number of cells g^{-1} of tissue (Fig. 1). For classification of samples as either positive or negative, there was some agreement between assays (Table 4) with 37% of samples classified as positive by both assays while 9% of samples were classified as negative by both assays.

To evaluate how use of the spiked water standard curve influenced correlation analysis, the equation of the line for gDNA spiked into kDNA was calculated using mean Cq values for these runs. This equation was then used to estimate cell counts for each positive sample including ones classified as below the QL. The data were then \log_{10} transformed and plot-

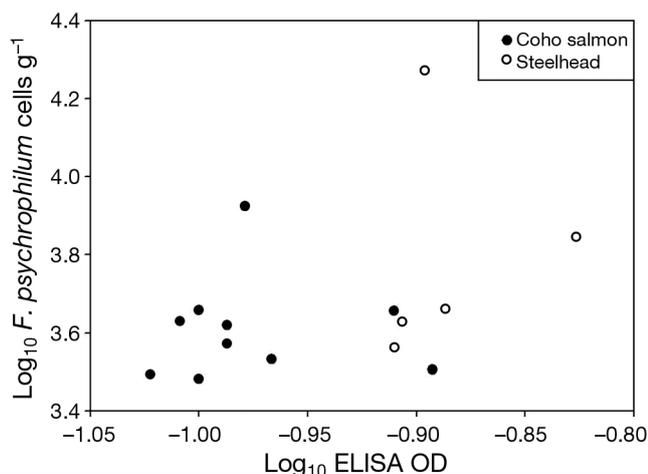


Fig. 1. \log_{10} -transformed ELISA OD values versus estimated number of *Flavobacterium psychrophilum* cells g^{-1} kidney tissue. Steelhead *Oncorhynchus mykiss* (n = 5) and coho salmon *O. kisutch* (n = 10) samples were included in the analysis if they were both positive by ELISA and had a Cq value ≥ 37 , the quantifiable limit of the qPCR assay. There was no correlation between ELISA and qPCR values ($r_s = 0.42$; $p > 0.05$) for these samples

Table 4. Cross-tabulated results for steelhead *Oncorhynchus mykiss* and coho salmon *O. kisutch* kidney tissue samples screened by quantitative PCR and ELISA for the presence of *Flavobacterium psychrophilum*

ELISA	qPCR	Steelhead	Coho salmon	Total
POS	POS	7	33	40
POS	NEG	4	22	26
NEG	POS	32	0	32
NEG	NEG	10	0	10
	Total	53	55	108

ted against the transformed ELISA OD values. As expected, estimated cell counts increased but the correlation between the 2 assays was still minimal with an r_s value below 0.10 (data not shown).

DISCUSSION

The qPCR assay developed for *fp1493* was both sensitive and specific to *Flavobacterium psychrophilum*. Primers for *fp1493* did not amplify other common *Flavobacterium* spp. or other fish pathogens. Other *F. psychrophilum* strains have not been tested by this assay; however, Lindstrom et al. (2009) demonstrated that MAb FL43 is reactive to 67 different *F. psychrophilum* strains, consistent with conservation of *fp1493*. The assay was also reproducible between replicates and runs as indicated by the low CV values.

The qPCR assay consistently detected as few as 8 *F. psychrophilum* cells in 2 diluents, sterile water and DNA extracted from fish kidney. The difference between mean Cq for the standard curve in sterile water versus kDNA increased as copy number decreased. Overall, the difference in mean Cq values between the 2 ranged from 0.5 to 2 cycles. A decrease in efficiency was also observed when kDNA was used as the diluent. These results may be due to the presence of inhibitors such as heme or the kDNA itself, which is at a high concentration even after being diluted 1:10 in water. Water was used as the diluent for standard curves run with samples because of the greater consistency and burbot kidney availability was limited. Furthermore, this is a common practice when performing absolute quantification of pathogen load in tissue samples (Powell et al. 2005, Chase et al. 2006, Griffin et al. 2008, Orioux et al. 2011, Sandell & Jacobson 2011). This practice did not affect the level of agreement between assays because samples that did not amplify or those with mean Cq values ≥ 37 were classified as negative.

Previous studies comparing polyclonal ELISA to qPCR for detection of *Renibacterium salmoninarum* have generally found strong agreement between the 2 (Powell et al. 2005, Chase et al. 2006, Jansson et al. 2008, Sandell & Jacobson 2011). Agreement between assays was not observed in this study, which is not surprising given that previous studies with *F. psychrophilum* have been unable to relate CFU ml⁻¹ to ELISA OD values (Long et al. 2012). The lack of correlation between the 2 assays may be due in part to the monoclonal antibody used in the ELISA. This is not the first report of low level of agreement between qPCR and a monoclonal-based ELISA (Jansson et al. 2008).

Another factor likely influencing agreement between the 2 assays is FP1493 itself. This outer membrane protein may not be secreted, in contrast to p57 from *R. salmoninarum*, which is secreted into surrounding tissue (Rockey et al. 1991). However, FP1493 may be up- or downregulated under different environmental conditions. Evidence suggests that FP1493 plays a role in iron acquisition for *F. psychrophilum* (Gliniewicz et al. 2012), and it has minor homology to transport proteins such as Por and HmuY protein. HmuY from *Porphyromonas gingivalis* is a heme-binding lipoprotein found on the outer membrane that acts in conjunction with other proteins to transport heme molecules into the bacterial cell (Olczak et al. 2008). The HmuY promoter is regulated by iron levels and is more active when iron is limited (Olczak et al. 2008), i.e. in the host environment, which results in higher levels of protein expression. When *F. psychrophilum* is grown *in vivo* or in iron-limited medium, FP1493 spot intensity is increased as detected by 2-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (LaFrentz et al. 2009). Additionally, increased presence of FP1493 on the outer membrane of the cell results in increased ELISA OD values when *F. psychrophilum* is grown in iron-limited media (Long et al. 2012).

F. psychrophilum was detected by qPCR in samples that had been classified as negative by ELISA in the current study, an outcome that has been reported previously for other fish pathogens (Chase et al. 2006, Jansson et al. 2008, Sandell & Jacobson 2011). Conversely, the ELISA detected *F. psychrophilum* in 23 kidney tissue samples from coho salmon that were classified as negative by qPCR. The detection by ELISA of *F. psychrophilum* antigens in kidney tissues from a majority of coho salmon sampled from the same hatchery in multiple, non-consecutive years (Lindstrom et al. 2009, Long et al. 2012) suggests that

antigen levels in the tissue may be high even when bacterial populations are low. Whether this is due to over-expression of *fp1493* or persistence of the antigen in tissues is unknown. *R. salmoninarum* antigens can persist in tissues for more than 110 d (Pascho et al. 1997). It is unlikely that FP1493 persists in tissues if it is not secreted and previous challenge experiments have shown a decrease in ELISA OD values with time (Long et al. 2012), but its persistence time-frame is unknown. Finally, there is considerable difference in the amount of tissue used for the 2 assays, 25 mg (qPCR) as compared to 500 mg (ELISA), and distribution of bacteria in kidney tissue can be spatially heterogeneous. If it had been feasible to extract a larger mass of tissue for the qPCR assay, it is possible that more samples would have been positive.

In a recent study, Long et al. (2012) used 4 diagnostic assays (bacteriological culture, nested PCR, membrane-filtration fluorescent antibody test, and ELISA) to screen spawning female salmonid broodstock for *F. psychrophilum*. Data generated from that study along with the current study indicates that *F. psychrophilum* prevalence in broodstock is higher overall at facilities rearing anadromous species as compared to selected rainbow trout facilities (Long et al. 2012). Nevertheless, more samples from multiple rainbow trout facilities are needed to determine if this can be generalized. Differences in the distribution of bacteria in various host species have also been reported for salmonid stocks in the Laurentian Great Lakes screened for *R. salmoninarum* (Faisal & Eissa 2009). Prevalence differences between species and facilities may be a function of the host reproductive strategy (semelparous versus iteroparous), different biosecurity measures taken at individual facilities, differences in immune response between host species, or, in the case of *F. psychrophilum*, a result of the different genetic lineages (Soule et al. 2005).

In conclusion, we have successfully developed a specific quantitative PCR assay for use in screening kidney tissue samples for *F. psychrophilum*. Although we were unable to show a correlation between ELISA OD values and qPCR results in spawning broodstock, this does not necessarily mean these assays cannot be used to screen this population. Rather, the data indicate that the ELISA with MAb FL43 may be a better indicator of antigen expression and possibly virulence. The qPCR assay can be used to determine bacterial load in tissue and potentially environmental samples. Studies are currently underway to determine if this assay can be successfully adapted for use with ovarian fluid. If so, this assay may be used to better quantify bacterial concentra-

tion in ovarian fluid, which is not possible by ELISA, and therefore increase our understanding of vertical transmission of *F. psychrophilum*. These data will inform management strategies for reduction and prevention of BCWD outbreaks.

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