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Comparison and evaluation of *Renibacterium salmoninarum* quantitative PCR diagnostic assays using field samples of Chinook and coho salmon

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ABSTRACT: *Renibacterium salmoninarum* is a Gram-positive bacterium causing bacterial kidney disease (BKD) in susceptible salmonid fishes. Several quantitative PCR (qPCR) assays to measure *R. salmoninarum* infection intensity have been reported, but comparison and evaluation of these assays has been limited. Here, we compared 3 qPCR primer/probe sets for detection of *R. salmoninarum* in field samples of naturally exposed Chinook and coho salmon first identified as positive by nested PCR (nPCR). Additional samples from a hatchery population of Chinook salmon with BKD were included to serve as strong positive controls. The 3 qPCR assays targeted either the multiple copy major soluble antigen (*msa*) genes or the single copy *abc* gene. The *msa*/non-fluorescent quencher (NFQ) assay amplified *R. salmoninarum* DNA in 53.2% of the nPCR positive samples, whereas the *abc*/NFQ assay amplified 21.8% of the samples and the *abc*/TAMRA assay 18.2%. The enzyme-linked immunosorbent assay (ELISA) successfully quantified only 16.4% of the nPCR positive samples. Although the *msa*/NFQ assay amplified a greater proportion of nPCR positive samples, the *abc*/NFQ assay better amplified those samples with medium and high ELISA values. A comparison of the geometric mean quantity ratios highlighted limitations of the assays, and the *abc*/NFQ assay strongly amplified some samples that were negative in other tests, in contrast to its performance among the sample group as a whole. These data demonstrate that both the *msa*/NFQ and *abc*/NFQ qPCR assays are specific and effective at higher infection levels and outperform the ELISA. However, most pathogen studies will continue to require multiple assays to both detect and quantify *R. salmoninarum* infection.

KEY WORDS: *Renibacterium salmoninarum* · Quantitative PCR · Assay comparison · Natural infection

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INTRODUCTION

Renibacterium salmoninarum infection is common among free-ranging and captive salmonid populations and causes bacterial kidney disease (BKD) in susceptible fish. BKD typically causes a chronic disease but can also lead to high mortality, particularly in high density rearing situations. The bacterium can be transmitted either vertically from infected females or horizontally (Balfry et al. 1996, McKibben & Pascho 1999). Transmission from hatchery to naturally reproducing populations, and vice versa, has been documented (Mitchum & Sherman 1981). As efforts at controlling *R. salmoninarum* via vaccination have met with limited success in Pacific salmon (Kaattari & Piganelli 1997, Piganelli et al. 1999, Alcorn et al. 2005), antibiotic treatment and broodstock culling of highly infected egg lots at spawning are the primary methods for mitigating the disease. Thus, efforts to control the spread of the pathogen require the availability of sensitive and reliable detection assays.

Bacterial culture has been a standard assay for many years due to its sensitivity and because a positive result indicates live *Renibacterium salmoninarum* cells, in-

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stead of cellular products, such as the major soluble antigen (msa), that may persist after cell death (Pascho et al. 1997). However, while the reagents used in this procedure are inexpensive, it is time consuming (plates need to be cultured for several weeks before *R. salmoninarum* colonies are detected) and does not lend itself well to large sampling studies, such as broodstock screening efforts at salmon hatcheries. In addition, a number of reports in the literature suggest that newer assays may provide greater sensitivity and faster results (Pascho & Mulcahy 1987, Gudmundsdóttir et al. 1993, Jansson et al. 1996, Miriam et al. 1997).

A number of non-culture diagnostic tests have been developed that target either *Renibacterium salmoninarum* antigens or nucleic acids. The enzyme-linked immunosorbent assay (ELISA) has been widely used at salmon hatcheries in the Pacific Northwest (USA) for broodstock screening for *R. salmoninarum* due to its ability to test many samples simultaneously and to quantify the level of bacterial antigen. The ELISA also has drawbacks, including limited sensitivity, cross-reactivity (Wood et al. 1995, Kim et al. 2007), and variability between antibody lots that can make data comparison between laboratories difficult. For these reasons, nested PCR (nPCR) (Chase & Pascho 1998) has become widely used for the detection of *R. salmoninarum* outside of the hatcheries. Though extremely sensitive, nPCR does not supply a quantitative output of infection intensity and may detect DNA from non-viable bacteria (Josephson et al. 1993). It is also prone to false positives because the amplified product (an *msa* gene segment) poses a contamination risk in ongoing assays.

Newer molecular techniques, such as reverse-transcriptase PCR (RT-PCR), detect and amplify recently transcribed gene products (RNA), thus only quantifying an active infection (although detection of mRNA does not necessarily indicate protein translation). However, this requires the isolation and preservation of RNA, which can be difficult in the field, and adds the cost and effort of validating a 2-step reaction process, first to convert mRNA to cDNA, and then to amplify the cDNA using a standard quantitative PCR (qPCR). Standard DNA-based qPCR can detect non-viable bacterial DNA, as can the nPCR, but it offers other advantages. The DNA-based qPCR allows the detection and quantification of the number of target genes in a sample ('mean quantity') with easier sample storage and preparation, a short processing time (in comparison to culture or the fluorescent antibody technique, FAT), and the ability to quickly analyze a large number of samples. Recently, a number of DNA- and RNA-based qPCR assays have been reported (Powell et al. 2005, Chase et al. 2006, Bruno et al. 2007, Suzuki & Sakai 2007, Jansson et al. 2008, Halaihel et al. 2009). To date, however, there have been no direct comparisons of these various qPCR assays for the detection of *Renibacterium salmoninarum* in Chinook *Oncorhynchus tshawytscha* and coho *O. kisutch* salmon, and only limited comparison with other standard diagnostic tests (e.g. ELISA or nPCR).

The qPCR assays used in this study target either the multiple copy *msa* genes (Chase et al. 2006) or the single copy *abc* transporter permease (*abc*) gene (Rhodes et al. 2006) of *Renibacterium salmoninarum*. The 2 assays that target the *abc* gene differ in probe chemistry. The first uses a standard TAMRA probe quencher while the other utilizes a non-fluorescent quencher (NFQ) and a minor groove-binding (MGB) linker that binds to the double-stranded DNA helix between the phosphate-sugar backbones. The major goal of this study was to evaluate the *abc* qPCR assay and to compare it with the *msa* qPCR using juvenile Chinook and coho salmon naturally infected with *R. salmoninarum*, as well as some heavily infected hatchery fish that served as strong positives. Here, we report the comparative specificity, repeatability and reproducibility of the *R. salmoninarum* qPCR assays, including evaluation against the widely used *R. salmoninarum* ELISA assay, which provided an indication of infection severity, and the nPCR as the reference measure of *R. salmoninarum* exposure. This study utilized guidelines set forth by the World Organization for Animal Health (Belak & Thoren 2006) for assay validation. Additionally, Bustin et al. (2009) presented a framework for nomenclature and standardized reporting with regard to qPCR assays to avoid confusion; we have incorporated these suggestions where possible. The results presented here will aid other researchers in choosing among these techniques for *R. salmoninarum* detection and quantification in future research.

MATERIALS AND METHODS

Sample collection and DNA extraction. The majority of fish used in this study were juvenile Chinook and coho salmon captured in the nearshore off the coasts of Oregon and Washington. Immediately following capture via surface trawl, the fish were sorted, placed on ice, identified to species and given a unique sample number. Fish were then frozen at −80°C until a later date, when necropsies were performed and the anterior kidney excised, placed in a 2 oz (5.67 g) Whirlpak bag, and refrozen at −80°C (the posterior kidneys were removed for *Nanophyetus salmincola* analysis). During necropsy, instruments were placed in a 20% bleach solution between fish for at least 2 min and rinsed twice in water to prevent cross-contamination of DNA samples.

A second group of juvenile Chinook salmon (n = 18) were obtained from the Oregon Department of Fish and Wildlife (ODFW) Cole River Hatchery (CRH) (Trail, Oregon). These fish were provided by the ODFW Fish Health Unit (Corvallis, Oregon) and were sampled as mortalities at the hatchery; kidneys were removed and stored at −20°C until analyzed as described below. Most of the CRH kidneys had white pustules indicative of clinical BKD.

Anterior kidney samples were thawed and homogenized with a rolling pin while still in the Whirlpak bag; 25 mg of kidney tissue (±2.5 mg) was removed with a sterile plastic spatula and placed into a 1.5 ml microcentrifuge tube. The work area and rolling pin were frequently bleached with a 20% $NaClO_3$ solution and care was taken to prevent cross-contamination of samples. Suggested guidelines from the American Fisheries Society Blue Book for quality assurance/quality control were followed (AFS-FHS 2007). Kidney tissues from black rockfish *Sebastes melanops* were included with each set of DNA extractions (36 samples per set) as a negative extraction control. DNA extraction was conducted using the DNeasy Tissue Kit (single tubes) following the manufacturer's (Qiagen) instructions for Gram-positive bacteria. DNA was eluted with 200 µl of Buffer AE heated to 70°C. Following elution, samples were either run immediately or stored at −80°C until analyzed.

Nested PCR (nPCR). To detect the presence of *Renibacterium salmoninarum,* all samples were initially tested via the nPCR protocol of Chase & Pascho (1998). The thermocycling regime was slightly modified to optimize results on our thermocycler, an MJ Research P100, as follows: 88°C for 3 minutes, 94°C for 2 min, then 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min. Reagent concentrations and primers were unchanged. The protocol was also modified to allow the use of 96-well PCR plates (Fisher Scientific #05-500-48) after a trial was conducted to test assay sensitivity using several different brands of PCR plates (data not shown). DNA concentration was not adjusted prior to nPCR analysis. All plates contained positive controls, no template controls, and the *R. salmoninarum*-negative DNA extraction controls. Amplified samples were subjected to electrophoresis on 1% agarose gels and visualized by staining with ethidium bromide, then photographed under UV light. Only samples testing positive with nPCR, and negative extraction controls, were further analyzed by ELISA and qPCR. A total of 220 DNA samples containing *R. salmoninarum* DNA detected by nPCR were used in this study (including 18 CRH samples).

Enzyme-linked immunosorbent assay (ELISA). Samples that were positive with the nPCR were tested for *Renibacterium salmoninarum* antigen by the ELISA II method (Pascho & Mulcahy 1987), a widely used standard reference assay for quantifying the degree of *R. salmoninarum* infection. The resulting optical densities (OD) were binned into 4 categories: background (OD < 0.08), low (0.081 < OD < 0.199), medium (0.199 < OD < 0.499), and high (OD > 0.499).

Quantitative PCR. All qPCR primer and probes sequences are provided in Table 1. Initially, we compared 2 different probes for the *abc* qPCR assays. The first *abc* probe was labeled with a 5′ FAM reporter and 3′ TAMRA quencher as developed by Rhodes et al. (2006). The second probe was modified to contain a MGB linker and a non-fluorescent quencher. The *abc* primers were not modified from Rhodes et al. (2006). The *msa* assay was performed as reported by Chase et al. (2006) but the probe was also modified with a MGB linker and NFQ quencher. Plasmid DNA encoding either the *abc* or *msa* genes were used as absolute standards (the *abc* plasmid was supplied by L. Rhodes; the *msa* plasmid was supplied by K. True). The plasmid DNA was diluted in 10-fold increments in a range of 10^8 to 10^1 copies and used to create a standard curve. Fresh standards were made each day from a concentrated stock stored at −80°C (Rhodes et al. 2006). Prior to the qPCR, DNA was quantified using the PicoGreen assay (Invitrogen) using a Victor plate fluorometer (Perkin Elmer). Since the samples consisted of DNA

Table 1. Primer and probe sequences used for qPCR detection of *Renibacterium salmoninarum*. Note that some of the probes used in this study utilized a minor groove-binding (MGB) linker with a non-fluorescent quencher (NFQ) that differ from those used in the original publications

qPCR target	Name	Sequence (5′ to 3′)	Source
msa genes	RS1238	GTG ACC AAC ACC CAG ATA TCC A	Chase et al. (2006)
msa gene	RS1307	TCG CCA GAC CAC CAT TTA CC	Chase et al. (2006)
msa genes	RS1262.NFQ probe[a]	FAM-CAC CAG ATG GAG CAA C-MGB/NFQ	Chase et al. (2006)
abc gene	ABCtransfor2	CTA AAC GAT TTC CCG GTC AA	Rhodes et al. (2006)
abc gene	ABCtransrev2	GAT TTT GCC TGC TGG TAT TTC C	Rhodes et al. (2006)
abc gene	ABCtrans probe	FAM-AAG CGC CAG CAG TCG ACG GC-TAMRA	Rhodes et al. (2006)
abc gene	ABC.NFQ probe[a]	FAM-AAG CGC CAG CAG TCG ACG GC-MGB/NFQ	Rhodes et al. (2006)

[a]In the original publication, 3′ end of the probe was labeled with TAMRA

extractions from equal weights of kidney tissue, we originally assumed that they would have roughly equivalent amounts of DNA present. However, DNA concentration varied widely following elution from the Qiagen DNeasy kits (data not shown). To ensure that equal amounts of DNA were present in each reaction, samples with low DNA yields were concentrated using a speed vacuum procedure (volume was reduced by 2/3) and all DNA samples were normalized to 20 ng μl^{-1} using a Biomek FX robotic workstation (Beckman Coulter). All experimental samples, negative DNA extraction controls and standards were run in duplicate wells; 4 wells of no template controls (NTC) were included on each plate. Quantitative PCRs were conducted using the Applied Biosystems (ABI) 7500 instrument and data analyzed with the sequence detection software (V.1.22). All dual-labeled probes and the Taqman Universal Master Mix, qPCR plates and optical covers were purchased from ABI; primers were purchased from Integrated DNA Technologies. All assays contained 900 nM primers, 200 nM probe and a total of 100 ng template DNA.

Samples with a 'quantification cycle (C_q) value' (Bustin et al. 2009) over 38 were considered negative because amplification in this range is near the theoretical lower limit of detection (less than 10 copies of the genome in the sample) (Note: 'C_q' is referred to as 'C_T' in ABI software). Each time a segment of target DNA is amplified in the qPCR assay, a sequence-specific probe begins to fluoresce as the probe quencher (NFQ) is deactivated; thus the level of fluorescence is correlated to the amount of target DNA in the sample. Samples with lower initial target DNA concentrations require more amplification cycles to generate enough fluorescence to cross the threshold quantification value; thus, for the qPCR, a higher C_q value is indicative of a lower initial target DNA concentration.

Evaluation of specificity, repeatability and reproducibility. An initial evaluation of the sensitivity of the *abc*/TAMRA and *abc*/NFQ qPCR assays was done by comparing the plasmid standard curves and reaction efficiencies. To test the analytical specificity of the *abc* qPCR assays, we extracted DNA from putative uninfected coho salmon, Chinook salmon and black rockfish (the salmon samples were from disease-free populations and were negative in repeated tests with nPCR). Seed stocks of phylogenetically related bacteria or common bacterial fish pathogens (see Table 2) were cultured on American Type Culture Collection (ATCC)-recommended agar and harvested via scraping and sterile water rinse. The wash from 3 plates of each species were combined and centrifuged for 10 min at 12 000 × *g*; the pellet was then divided into 5 microcentrifuge tubes and digested in lysis buffer. DNA extraction proceeded as for the study samples using Qiagen protocols (see above), and the DNA concentration was normalized to 20 ng ml^{-1} prior to qPCR. *Renibacterium salmoninarum* (ATCC 33209) served as a positive control.

Repeatability ('intra-assay variance') (Bustin et al. 2009) and reproducibility ('inter-assay variance') of the qPCRs was tested by comparing replicates of the same samples. Select samples with adequate DNA quantities (n = 6) were run in 4 to 8 replicate wells on the same qPCR plate to test intra-assay variance. This procedure was repeated on 3 independent assay plates to test inter-assay variability on consecutive days.

Statistical analysis. Calculations of C_q value and mean quantity, as well as standard deviations, were generated by the ABI software provided with the ABI 7500 optical thermocycler. The mean quantity is the number of target genes amplified in the sample, derived by comparing the C_q value of a sample with the standard curve, which contains known numbers of the target gene. Thus, for a single copy target gene the mean quantity is a direct estimate of the number of bacteria present in the sample. Calculations of geometric mean quantities and the coefficient of variation (CV: the ratio of the standard deviation to the mean) were done in Microsoft Excel. Repeatability comparisons of differences in the C_q values were performed using the non-parametric Sign test. Comparisons of C_q values for sample reproducibility (between plates) were performed using the Kruskal-Wallis nonparametric ANOVA because of the non-normal distribution of these data. Differences in the detection performance of the qPCR assays were compared with a chi-square test using StatView software (v. 1998, SAS Institute). In all cases, significance was defined as $p \leq 0.05$.

Visual data comparisons and regression r^2 values were calculated using Tableau software (v. 2008, Tableau Software). Samples which did not amplify were arbitrarily assigned a C_q of 42 for clarification, since the C_q has an inverse relationship with the quantity of gene product present (i.e. a 'low' C_q indicates the presence of more gene copies than a 'high' value). Throughout this paper we refer to samples falling within the given ranges as having the following C_q 'scores': 'high' ($14 < C_q < 20$), 'medium' ($20.01 < C_q < 30$), and 'low' ($30.01 < C_q < 38$).

RESULTS

Initial sample selection

A total of 202 samples were classified as positive for *Renibacterium salmoninarum* by nPCR and were included in this qPCR evaluation study. We also

obtained kidney samples from 18 Chinook salmon with clinical signs of BKD from the CRH (ODFW), all of which tested positive by nPCR, providing a total of 220 nPCR positive samples to be compared in further analyses.

ELISA

The ELISA detected quantifiable levels of *Renibacterium salmoninarum* antigen in 16.4% of the samples previously determined to be positive by the nPCR (n = 36/220). Of the ELISA positives, we binned 13.9% as highly infected (OD > 0.499), 2.7% as moderately infected (0.199 < OD < 0.499), and 0.9% as having a low infection level (0.081 < OD < 0.199); 82.5% were at background levels (OD < 0.08). All of the qPCR assays amplified the majority of the ELISA positive samples. The *abc*/NFQ qPCR identified 88.9% (32/36) as positive, the *abc*/TAMRA qPCR detected 72.2% (26/36), and the *msa*/NFQ qPCR detected 94.4% (34/36). All but 2 of the CRH samples were binned as highly infected by ELISA; the remaining 2 were at background OD levels. The ELISA detected 5 samples (from the naturally infected salmon) as positive that were not amplified by any of the 3 qPCR assays ('unique positives'): 2 of these were in the low ELISA range, 1 was medium, and 2 were in the high range. All of the negative controls were also negative with the ELISA.

qPCR specificity

Initial tests showed that the 2 *abc*/NFQ qPCR primer/probe sets did not amplify either Chinook or coho salmon *Renibacterium salmoninarum*-negative DNA, any of the 13 other bacterial species tested (Table 2), or any of the negative controls.

qPCR repeatability and reproducibility

There was no statistical difference either within or among the plates in the repeatability and reproducibility study for either C_q values or geometric mean quantities (see Appendix 1). Although there were differences in the quantities of the *abc* gene detected between the 6 different samples, these were consistent for each sample.

Table 2. Bacterial species tested to assess the specificity of the *abc* gene quantitative PCRs, which differed in the probe quenchers (one utilized a TAMRA probe while the other used a non-fluorescent quencher, NFQ). Positive amplification (+) and no amplification (–) are indicated. ATCC: American Type Culture Collection, Manassas, Virginia

Species	Source	*abc*/ TAMRA	*abc*/ NFQ	*msa*/ NFQ
Renibacterium salmoninarum	ATCC 33209	+	+	+
Phylogenetically similar				
Arthrobacter globiformis	ATCC 8010	–	–	–
Corynebacterium aquaticum	ATCC 14665	–	–	–
Micrococcus [*K.*] *varians*	ATCC 15306	–	–	–
Micrococcus luteus	ATCC 4698	–	–	–
Other pathogenic bacteria				
Aeromonas hydrophila	ATCC 7966	–	–	–
Aeromonas salmonicida	ATCC 33658	–	–	–
Edwardsiella tarda	ATCC 15947	–	–	–
Flavobacterium columnare	ATCC 43622	–	–	–
Flavobacterium psychrophilum	ATCC 49418	–	–	–
Pseudomonas fluorescens	ATCC 13525	–	–	–
Listonella ordalii	ATCC 33509	–	–	–
Listonella anguillarum	ATCC 68664	–	–	–
Yersinia ruckeri	ATCC 29473	–	–	–

Preliminary comparison of the qPCR assays

The samples analyzed with each of the 3 primer/probe sets were run on 5 qPCR plates. Mean reaction efficiencies ($E = 10^{(-1/\text{slope})} - 1$) for the 3 assays were all similar and approximately 100%. Regression values from the standard curves exceeded 0.99 for all assays. Of the 202 samples detected as positive by the nPCR, 44.5% were negative in all 3 qPCR assays (n = 98).

The *msa*/NFQ qPCR was the most sensitive of the 3 assays, detecting 53.2% (n = 117) of the samples testing positive with the nPCR (Table 3). Many of these were near the limit of detection, which resulted in the *msa*/NFQ qPCR assay having the highest average C_q value (32) and the highest percentage of non-reproducible amplification (samples detected in only 1 of the 2 wells; 34.2%) (Table 3). A majority (63%) of the samples amplified by this assay had a C_q value in the range of 35 to 38; the mean quantity of the *msa*

Table 3. Comparison of the percentage of samples detected, mean quantification cycle (C_q) values, and coefficients of variation for the 3 qPCR assays. Both field samples (n = 202) and Cole River Hatchery fish (n = 18) are included

Summary	*abc*/ NFQ	*abc*/ TAMRA	*msa*/ NFQ
% positive by the assay	**21.8**	**18.2**	**53.2**
% with only 1 well detected	14.6	17.5	34.2
Average C_q	24.6	27.4	32.0
Coefficient of variation (CV)	20.1	26.3	28.7

gene detected in these samples was very low (5.17). With all of the positive samples included, the *msa*/NFQ assay had the second highest mean quantity (1.5×10^2) and the highest CV (28.7).

The *abc*/NFQ qPCR detected 21.8% (n = 48) of the nPCR positive samples, but had the lowest average C_q value (24.6), the highest geometric mean quantity (4.1×10^3) and the lowest CV (20.1) of the 3 assays. It also had the lowest number of samples with non-reproducible amplification (14.6%). Fewer samples were quantified in the C_q range of 35 to 38 (20.8%).

The *abc*/TAMRA assay detected only 18.2% of the nPCR positive samples (n = 40) and had an average C_q value of 27.4. This assay produced the lowest geometric mean quantity (138.4) of the 3 assays, and fell between the others with regard to CV (26.3) and the percentage of non-reproducible amplifications (17.5%) (Table 3). All CRH Chinook salmon samples had quantifiable *Renibacterium salmoninarum* DNA levels in the *msa*/NFQ and *abc*/NFQ assays, but only 77.8% of the samples amplified with the *abc*/TAMRA assay.

Overall, the *msa*/NFQ assay detected 65 samples that tested negative with the *abc*/NFQ, while the *abc* assay detected 2 samples that tested negative with the *msa*/NFQ qPCR. A chi-square comparison of the 2 qPCR assays indicated that the *msa*/NFQ detected significantly more positive samples than the *abc*/NFQ ($p < 0.001$) and the *abc*/TAMRA assays ($p < 0.001$). Based on these results, we selected only the *abc*/NFQ and *msa*/NFQ assays for further comparison.

Comparison of the qPCR results with ELISA

The qPCR assays all quantified more nPCR positive samples than the ELISA. The regression of average C_q value versus ELISA OD yielded an r^2 of 0.786 for the *abc*/NFQ assay and 0.903 for the *msa*/NFQ assay when all positive samples were included (Fig. 1a). However, when only those samples with a medium or higher ELISA OD were included in the regression analysis, the *abc*/NFQ assay r^2 was 0.727, while that of the *msa*/ NFQ assay declined to 0.579 (Fig. 1b). Of the samples that were negative by the ELISA (n = 184), 12% were positive with the *abc*/NFQ assay and 46.7% were positive with the *msa*/NFQ assay.

To further compare the ELISA and qPCR assays, we examined the ELISA OD values of samples that were binned into ranges of average C_q scores representing high ($C_q < 20$), medium ($20 < C_q < 30$) and low ($30 < C_q < 38$) bins (keep in mind that a lower C_q value corresponds with a higher gene target quantity, i.e. a more highly infected sample, while the ELISA OD directly correlates with the amount of bacterial antigen present). Only samples positive for both the *msa* and *abc* gene qPCRs were included. For the *abc*/NFQ assay, 31.2% of the samples in the high C_q range were also high by ELISA, while 14.6% were low (Table 4). Samples in the medium C_q bin were equally split in the ELISA bins, but samples in the low ELISA bin were almost evenly spread between the high (14.6%), medium (14.6%), and low (16.7%) C_q bins. For the *msa*/NFQ assay, a greater percentage of samples in the high and low C_q groups matched the corresponding ELISA value bins (Table 4) than for the *abc*/NFQ assay. Within the high C_q bin, all of the samples (15.4% of the total) had high ELISA values, and 70.1% of the samples with low C_q values also had low ELISA scores.

Comparative performance of the *abc*/NFQ and *msa*/NFQ qPCR assays

To test how well the 2 NFQ qPCR assays agreed with each other, the *abc*/NFQ and *msa*/NFQ C_q values were plotted against one another (Fig. 2). In general, samples with low C_q scores from both qPCR assays (mostly CRH) also had high ELISA values (OD = 1.71), while those with high C_q scores had a lower average OD (0.26). However, the assays were not always in agreement, and did not cluster on the regression line except near the limit of detection ($r^2 = 0.509$). This was largely due to the presence of 8 samples that were positive by the *abc*/NFQ assay ($C_q \approx 18$), but were close to the range of detection limit ($C_q \approx 35$) for the *msa*/NFQ and were below the background level for ELISA (average OD = 0.071). These samples are highlighted near the top of Fig. 2. To confirm these data, the 8 samples were rerun in the *abc*/NFQ assay and yielded similar results.

Table 4. Comparison of quantification threshold (C_q) and ELISA optical density (OD) values for the 2 *Renibacterium salmoninarum* specific qPCRs with non-fluorescent quencher (NFQ) probes. Samples were binned by C_q value; the percentage of samples in each bin within a certain ELISA OD range was then calculated. ELISA OD bins: low (<0.199); medium (0.199 to 0.499); high (>0.499); C_q scores: low (>30.01 to 38); medium (>20.01 to 30); high (<20)

	C_q score	ELISA OD			Row total (%)
		Background/low	Medium	High	
abc/NFQ	High	14.6	0	31.2	45.8
Total n = 48	Medium	14.6	0	12.5	27.1
	Low	16.7	4.2%	6.2	27.1
msa/NFQ	High	0	0	15.4	15.4
Total n = 117	Medium	3.4	0	5.1	8.5
	Low	70.1	4.3%	1.7	76.1

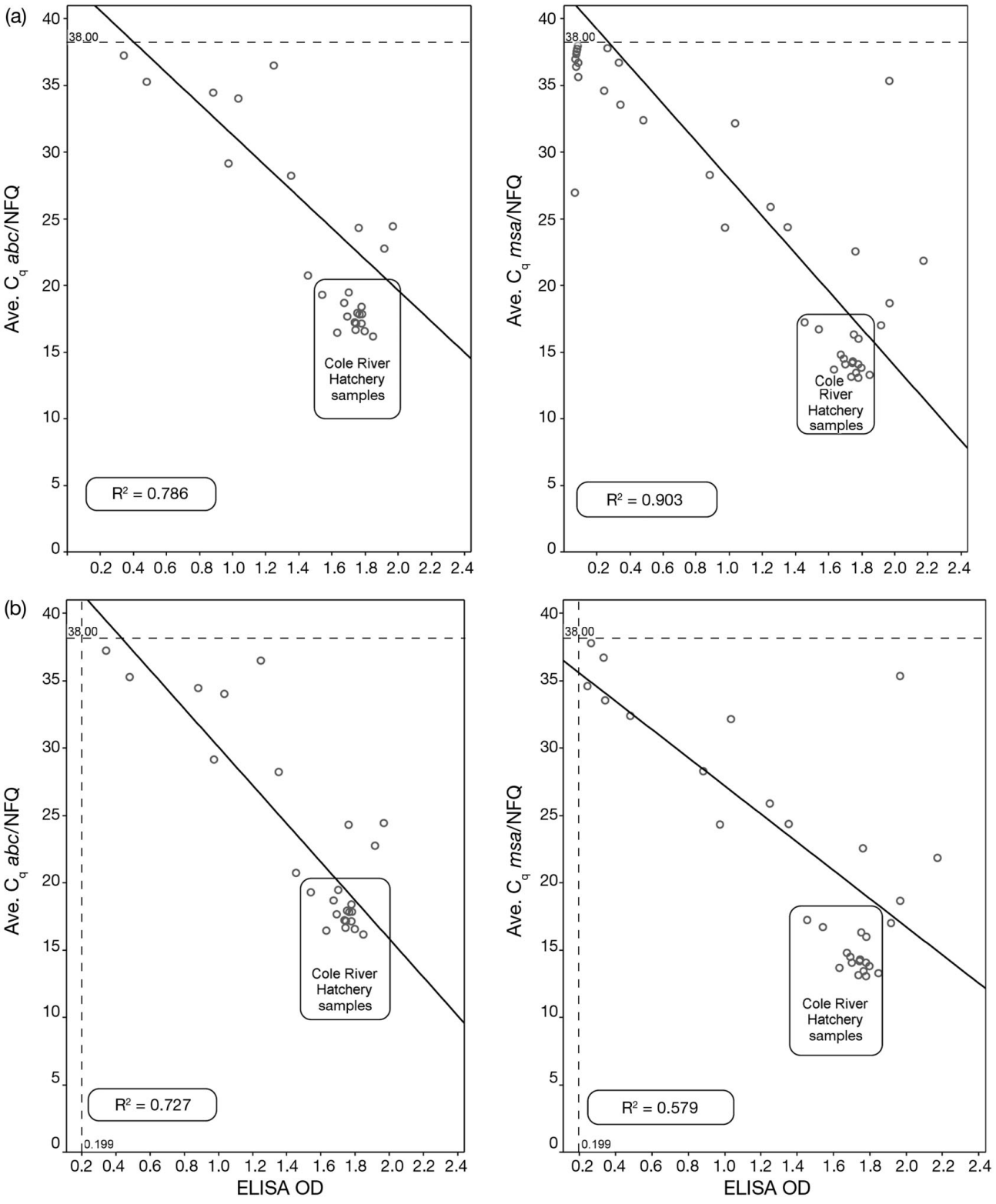

Fig. 1. Quantitative PCR *abc*/NFQ and *msa*/NFQ assay quantification cycle (C_q) values versus ELISA OD for (a) all positive samples, and (b) for those in the medium–high ELISA OD range only. The horizontal dashed lines indicate the C_q value limit of 38; the vertical dashed lines (b only) indicate the ELISA OD cutoff level (0.199) for medium and highly infected samples

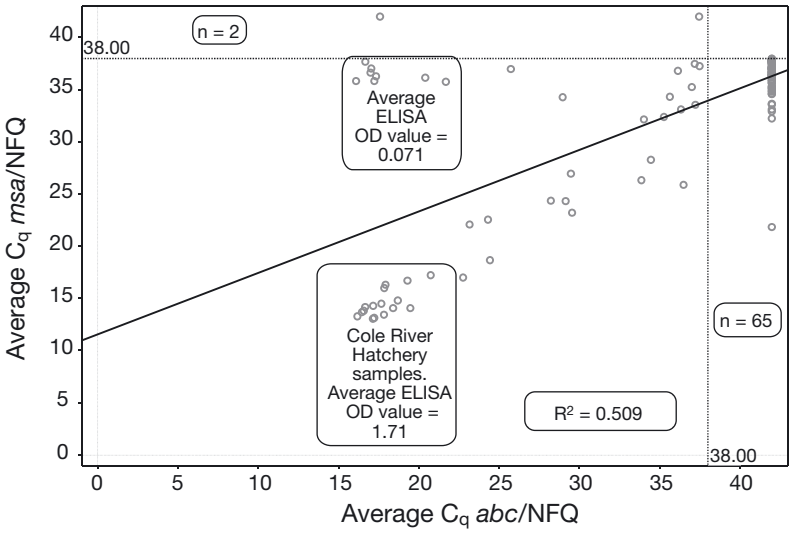

Fig. 2. A regression of *msa*/NFQ quantification cycle (C_q) values versus *abc*/NFQ values for all positive samples in the dataset. The horizontal and vertical dotted lines indicate the positive sample C_q value limit of 38. Average ELISA OD values for subgroups of the sample population are highlighted and the number of samples detected by only 1 of the 2 assays are noted beyond the dotted lines

Table 5. Comparison of geometric mean quantities (Qty), average quantification cycle (C_q) values, and their respective ratios for (a) all samples and (b) the Cole River Hatchery (CRH) samples only for both the *msa*/NFQ and the *abc*/NFQ qPCR assays. The ratios provide an indication of reaction efficiency among different subgroups of the sample set

	Geometric mean Qty	Average C_q	Quantity ratio (*msa*/*abc*)	C_q ratio (*msa*/*abc*)
(a) Samples that were positive by both assays, not including the CRH samples (n = 28)				
Avg. for *msa*/NFQ	234.3	31.5	0.48	1.133
Avg. for *abc*/NFQ	483.7	27.8		
Difference	–249.4	3.7		
(b) As for (a), including only the CRH samples (n = 18)				
Avg. for *msa*/NFQ	19.205.953.1	15.9	116.7	0.820
Avg. for *abc*/NFQ	164.597.4	19.4		
Difference	19.041.355.8	–3.48		

To further compare the *msa*/NFQ qPCR to the *abc*/NFQ PCR, we first divided the samples into 2 groups: samples positive in both qPCR assays, but without the CRH fish (Table 5a), and the CRH samples alone (Table 5b). We then calculated the geometric mean quantity ratio and C_q ratio (*msa*/NFQ value divided by *abc*/NFQ value in both cases) for each of these groups. These ratios provide a comparison of amplification efficiency across all levels of infection in the sample subpopulation. For the group with CRH samples excluded, the mean quantity ratio was 0.48 and the C_q ratio was 1.13; the *abc*/NFQ assay performed slightly better. When only the CRH samples were included, however, the mean quantity ratio increased to 116.7, while the C_q ratio decreased to 0.82; the *msa*/NFQ assay performed better among the very strong positives (CRH samples). These changes were driven by a large increase in the mean quantity detected by the *msa*/NFQ assay, which exceeded 1.9×10^7 for the CRH samples (Table 5b).

DISCUSSION

The *msa*/NFQ qPCR (Chase et al. 2006) was the most sensitive of the 3 qPCR assays, quantifying 53.2% of the *Renibacterium salmoninarum* nPCR positive samples (Table 3). Most of the samples (63%) detected by the *msa*/NFQ assay were near the lower limit of detection (C_q values in the range of 35 to 38). The *msa*/NFQ qPCR also had the highest percentage of samples detected in only 1 of the 2 wells (34.2%). Most of these non-reproducible amplifications were near the lower limit of detection (C_q value cutoff = 38), so the increased variability in this assay was coincident with increased sensitivity, as expected. The *msa*/NFQ assay was also in good agreement with the ELISA (Table 4), and had the highest r^2 value for the regression of average C_q versus ELISA OD when all positive samples were included (Fig. 1b). The higher sensitivity makes the *msa*/NFQ qPCR a good candidate assay for initial screenings of samples, especially when nPCR or culture will not be used and infection prevalence is a primary goal. However, because the *R. salmoninarum* genome contains at least 2 to 6 copies of the *msa* gene (Rhodes et al. 2004), the *msa*/NFQ assay mean quantity outputs may not directly correlate with the number of bacteria present in the sample. The assay would also be suitable for laboratory studies where the *R. salmoninarum* strain, and thus the number of *msa* copies, is known.

The *abc*/NFQ qPCR assay had excellent repeatability and did not cross-react with the bacteria in our screening panel or *Renibacterium salmoninarum*-

negative Chinook or coho salmon genomic DNA. The *abc*/NFQ qPCR detected only 21.8% of the samples that were positive by nPCR but had the highest geometric mean quantity and the lowest coefficient of variation (Table 3). It also had the highest average C_q value of the 3 assays, indicating that the *abc*/NFQ qPCR assay was better at amplifying samples with medium to high infection levels (based on ELISA values). The assay also performed better than the *msa*/NFQ qPCR for the regression of average C_q versus ELISA OD when only samples with medium to high ELISA values were included (Fig. 1b). This makes the *abc*/NFQ qPCR assay a good candidate for studies where initial prevalence of infection screening will be done via nPCR, and/or when detection of *R. salmoninarum* at infection levels that foreshadow the onset of bacterial kidney disease (based on comparison with the ELISA OD) is the prime concern. For this reason, it may be useful for hatchery broodstock screening programs because it identifies only those fish with medium to high levels of infection, without the drawbacks of the ELISA. The *abc*/NFQ assay is the best choice when genome quantification is the primary concern, because the *abc* primer set targets a single copy gene.

Some drawbacks with both the *abc*/NFQ and *msa*/NFQ qPCR assays were also identified. Neither of the qPCR assays amplified all of the samples previously identified as positive by nPCR. This was unexpected given the sensitivity reported by Chase et al. (2006), who also tested naturally infected fish. However, the juvenile Chinook salmon used in that study were all from a hatchery population and some had clinical signs of BKD, whereas the fish used in the present study were of unknown origin and had low level infections (at background infection levels with ELISA), with the exception of the CRH fish included to provide strong positive controls. In addition, the *abc*/NFQ assay strongly amplified a group of 8 samples (in the C_q range of 15 to 22) that were at background ELISA values and only weakly positive with the *msa*/NFQ qPCR (Fig. 1). The samples were re-tested and the results confirmed, but the possibility exists that the *abc*/NFQ qPCR is amplifying a similar gene from another bacterial species in the sample that was not included in our bacterial cross-reactivity panel (Table 2). Attempts at sequencing the product for clarification were unsuccessful and there was not enough DNA remaining in any of the 8 samples to pursue this avenue of investigation. A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed November 2009) did not produce any other closely aligned sequences for the *abc* gene which were not already included in our cross-reactivity study, although at present there are few fish pathogen sequences in the database.

Another issue was identified when the ratios of average C_q and geometric mean quantity for the 2 NFQ-based qPCR assays (*msa*/NFQ over *abc*/NFQ in all cases) were compared (Table 5). When the CRH samples were excluded, the *abc*/NFQ assay outperformed the *msa*/NFQ, yielding a slightly higher geometric mean quantity and higher average C_q score, with a C_q ratio of 1.13. When only the CRH samples were included, the *msa*/NFQ assay had a greatly increased geometric mean quantity (1.9×10^7) and the C_q ratio declined to 0.82, suggesting that it performed better among these samples, almost all of which had ELISA ODs > 1.2 (Table 5). The cause of this result is uncertain; the CRH samples were from heavily infected fish that were likely to contain *Renibacterium salmoninarum* strains with multiple *msa* gene copies, which could account for their virulence (Coady et al. 2006) and may explain the increase in amplification. However, log scale differences in gene quantity which may be attributable to multiple copies of the *msa* gene would still only result in minor changes in the resulting C_q value. These 2 assays are currently undergoing testing in fish experimentally injected with *R. salmoninarum* (thus with known infection levels), and that investigation may clarify these results (D. Elliott & M. Purcell pers. comm.).

This study was not designed to compare the nPCR to the 3 qPCR primer and probe sets. Instead of testing a mix of known positive and negative fish, we first screened the samples by nPCR because they were naturally exposed and their infection prevalence and severity were unknown. This precluded us from calculating either diagnostic sensitivity or specificity, as outlined in the World Organization for Animal Health (OIE) manual (Jacobson & Wright 2006). Under OIE guidelines, the ideal test of intra-assay variance would have been performed using true replicates from each fish, with each separate 25 mg kidney sample undergoing individual sample processing and DNA extraction. However, the small mass of kidney tissue available precluded that approach in this study, and the replicates we used were drawn from the same original sample of kidney tissue (i.e. 1 tube of DNA from each fish, rather than multiple tubes of DNA from each fish). The use of plasmids to serve as positive controls to generate standard curves in the qPCRs likely also resulted in an underestimation of the actual number of gene copies detected, as the plasmid amplifies more readily than genomic *Renibacterium salmoninarum* DNA (T. A. Sandell pers. obs.). However, the use of a plasmid that may be widely distributed among fish health laboratories provides a useful standard that removes the need for each laboratory to produce and quantify its own bacterial genomic DNA standards, removing a potential source of error.

CONCLUSIONS

Newer molecular techniques for the detection and quantification of pathogenic bacteria in fish are being developed, including DNA microarrays coupled with PCR (Call et al. 2003, Warsen et al. 2004), multiplex qPCR (at present, none have included *Renibacterium salmoninarum* in their panels; see Ji et al. 2004, Panangala et al. 2007), and the newer technique of loop-mediated isothermal amplification (Gahlawat et al. 2009). Eventually, a newly developed assay will surpass those currently available. However, without studies to evaluate and compare these protocols they are of limited use, and at present there is confusion about which assay is most appropriate and an inability to compare data between investigations.

Ideally, researchers in a given area (e.g. the Columbia River basin), or even a given region (e.g. the Pacific Northwest, USA), should be able to agree on using at least one common assay in all related studies, or to archive samples (or DNA) whenever possible for future comparative testing. This would immediately improve our understanding of how this pathogen affects the growth, survival and fitness of salmonid populations in regions where *Renibacterium salmoninarum* is widely endemic. At present, there is no 'gold standard' assay for the detection and quantification of *R. salmoninarum*; for example, other authors have also demonstrated that samples may be ELISA positive and nPCR negative (Faisal & Eissa 2009). While the present study is a step towards this goal, we are unable to recommend one molecular assay as the single best diagnostic assay for *R. salmoninarum*; as a result, 2 or more assays may be necessary to meet the goals of a specific project. However, both the *msa*/NFQ and *abc*/NFQ qPCR assays evaluated here do have utility in specific applications, as outlined above. It is our hope that this study will provide the first step in the selection of a common assay for *R. salmoninarum* detection and quantification.

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Appendix 1. Comparison of inter-plate (a) C_q values and (b) mean quantities for *abc* gene detection in repeatability tests of 6 nested PCR positive samples using the *abc* gene primers/MGB probe. p-values for significant differences between plates were calculated via the Kruskal-Wallis non-parametric ANOVA. GM: geometric mean; CV: coefficient of variation

Sample ID		n/plate	% positive	Plate 1 GM	Plate 1 CV	Plate 2 GM	Plate 2 CV	Plate 3 GM	Plate 3 CV	All 3 plates GM	All 3 plates CV	p
a) C_q												
2893	Sample 1	6	100	19.8	0.088	19.6	0.095	19.7	0.101	19.7	0.091	0.45
5332	Sample 2	8	100	35.8	0.017	34.4	0.088	33.8	0.082	34.6	0.069	0.83
5609	Sample 3	6	100	31.9	0.093	31.6	0.089	31.2	0.074	31.6	0.081	0.78
5697	Sample 4	6	100	19.9	0.027	19.7	0.028	19.7	0.027	19.7	0.026	0.72
5723	Sample 5	4	100	18.4	0.012	18.2	0.01	18.4	0.01	18.3	0.011	0.31
9249	Sample 6	6	100	19.9	0.103	19.6	0.1	19.8	0.102	19.7	0.096	0.62
b) Quantity												
2893	Sample 1	6	100	62490.7	1.04	62307.1	1.1	58753.5	1.16	61159.3	1.06	0.91
5332	Sample 2	8	100	1.0	0.395	1.6	1.6	3.3	1.42	1.5	1.79	0.22
5609	Sample 3	6	100	13.3	0.95	9.5	0.9	20.0	0.8	13.6	0.86	0.48
5697	Sample 4	6	100	61643.6	0.36	62019.1	0.38	62604.2	0.34	62087.7	0.34	0.99
5723	Sample 5	4	100	172320.9	0.15	185654.6	0.13	151541.2	0.12	169248.5	0.15	0.14
9249	Sample 6	6	100	57757.4	0.97	63593.0	1.02	55696.9	1	58922.7	0.94	0.86

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