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Bench-top validation testing of selected immunological and molecular *Renibacterium salmoninarum* diagnostic assays by comparison with quantitative bacteriological culture

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Abstract

No gold standard assay exhibiting error-free classification of results has been identified for detection of *Renibacterium salmoninarum*, the causative agent of salmonid bacterial kidney disease. Validation of diagnostic assays for *R. salmoninarum* has been hindered by its unique characteristics and biology, and difficulties in locating suitable populations of reference test animals. Infection status of fish in test populations is often unknown, and it is commonly assumed that the assay yielding the most positive results has the highest diagnostic accuracy, without consideration of misclassification of results. In this research, quantification of *R. salmoninarum* in samples by bacteriological culture provided a standardized measure of viable bacteria to evaluate analytical performance characteristics (sensitivity, specificity and repeatability) of non-culture assays in three matrices (phosphate-buffered saline, ovarian fluid and kidney tissue). Non-culture assays included polyclonal enzyme-linked immunosorbent assay (ELISA), direct smear fluorescent antibody technique (FAT), membrane-filtration FAT, nested polymerase chain reaction (nested PCR) and three real-time quantitative PCR assays. Injection challenge of specific pathogen-free Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), with *R. salmoninarum* was used to estimate diagnostic sensitivity and specificity. Results did not identify a single assay demonstrating the highest analytical and diagnostic performance characteristics, but revealed strengths and weaknesses of each test.

Keywords: bacteriological culture, diagnostic assays, enzyme-linked immunosorbent assay, fluorescent antibody technique, polymerase chain reaction, *Renibacterium salmoninarum*.

Introduction

Bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* is widespread in most areas of the world where wild or cultured salmonid fish are present, and several extensive reviews have been written on the pathogen and the disease (Fryer & Sanders 1981; Austin & Austin 1987; Elliott, Pascho & Bullock 1989; Evenden *et al.* 1993; Fryer & Lannan 1993; Pascho, Elliott & Chase 2002). This typically chronic disease can cause significant mortality in salmonids at most life stages in both fresh water and sea water. The aetiological agent of BKD is a small (0.3–1.5 μm by 0.1–1.0 μm), non-motile, non-spore-forming, non-acid-fast, slowly replicating, Gram-positive diplobacillus (Fryer & Sanders 1981) classified in the *Micrococcus-Arthrobacter* subdivision of the actinomycetes (Stackebrandt *et al.* 1988; Gutenberger *et al.* 1991). The bacterium can be transmitted horizontally in both fresh water (Mitchum & Sherman 1981; Bell, Higgs & Traxler 1984; Alcorn *et al.* 2005) and sea water (Murray *et al.* 1992; Evelyn 1993) as well as vertically from the female parent to progeny in association with the eggs (Bullock, Stuckey & Mulcahy 1978; Evelyn, Ketcheson & Prosperi-Porta 1984a; Evelyn, Prosperi-Porta & Ketcheson 1984b, 1986).

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Journal of Fish Diseases

Renibacterium salmoninarum also can survive and perhaps multiply within host macrophages (Young & Chapman 1978; Bruno 1986a; Bandín *et al.* 1993; Flaño *et al.* 1996; Gutenberger *et al.* 1997). Although BKD most frequently occurs as a systemic granulomatous disease, localized infections of the bacterium, with lesions confined to ocular or post-orbital tissues, brain or skin, have also been reported (Hendricks & Leek 1975; Hoffmann, Popp & van de Graaff 1984; Speare 1997; Ferguson 2006). Furthermore, *R. salmoninarum* may exist in some fish populations for prolonged periods as subclinical infections (Meyers *et al.* 1993a; Lovely *et al.* 1994; Starliper & Teska 1995; Jónsdóttir *et al.* 1998), and evidence suggests that infected fish do not inevitably succumb to disease, but can eliminate the pathogen under certain conditions (Sanders, Pilcher & Fryer 1978; Bruno 1986b; Lovely *et al.* 1994).

For many years, the unique characteristics of *R. salmoninarum* and its biology presented difficult obstacles to the development of reliable methods for the detection of infected fish (see review in Pascho *et al.* 2002). A major breakthrough in *R. salmoninarum* detection occurred with the successful culture of the bacterium on an artificial medium (Ordal & Earp 1956) and subsequent improvements to that medium (see Pascho *et al.* 2002). Bacteriological culture has been reported to be sensitive enough to support the growth of single *R. salmoninarum* cells (Evelyn 1977), but the extremely slow growth and fastidious nature of *R. salmoninarum* makes this method unsuitable for the rapid detection and quantification of the bacterium in field samples.

More recent advancements in *R. salmoninarum* diagnosis have resulted in the development of immunological and molecular methods that enable more rapid and specific detection of unique antigens and genome sequences of the bacterium (see Pascho *et al.* 2002). The principal non-culture methods currently in use include fluorescent antibody techniques (FAT), enzyme-linked immunosorbent assays (ELISA), and both non-quantitative and quantitative procedures for the polymerase chain reaction (PCR) (see e.g., Pascho *et al.* 2002; Powell *et al.* 2005; Chase, Elliott & Pascho 2006; Rhodes *et al.* 2006; Bruno *et al.* 2007; Suzuki & Sakai 2007; Jansson *et al.* 2008; Halaihel *et al.* 2009).

Research has indicated that rapid non-culture *R. salmoninarum* detection methods such as ELISA can aid fishery managers in developing more effective measures for monitoring and controlling BKD (Pascho, Elliott & Streufert 1991; Gudmundsdóttir *et al.* 2000; Meyers *et al.* 2003; Munson, Elliott & Johnson 2010). Because most of the immunological and molecular methods in widespread use for the identification of *R. salmoninarum* can detect nonviable as well as viable organisms (Elliott & Barila 1987; Miriam *et al.* 1997; Pascho, Goodrich & McKibben 1997; Cook & Lynch 1999; Suzuki & Sakai 2007), questions remain regarding the accuracy and biological significance of some *R. salmoninarum* detections based on such methods. In addition, a lack of standardized *R. salmoninarum* diagnostic tools, and frequent use of non-validated *R. salmoninarum* diagnostic methods that differ in specificity, sensitivity and reliability, has made it difficult to compare *R. salmoninarum* data collected by different fishery management agencies.

Validation is a process that determines the fitness for an intended purpose of an assay that has been properly developed, optimized and standardized. The validation process includes estimates of the analytical performance characteristics (analytical specificity and sensitivity and repeatability) and diagnostic performance characteristics (diagnostic specificity and sensitivity, and cut-off determination) for an assay (OIE 2009). These 'bench-top' determinations are followed by inter-laboratory testing to determine reproducibility and ruggedness of assay results (OIE 2009). Although comparisons of specificity and sensitivity among several non-culture methods for *R. salmoninarum* detection have been reported (see e.g., Pascho *et al.* 2002; Powell *et al.* 2005; Chase *et al.* 2006; Bruno *et al.* 2007; Suzuki & Sakai 2007; Jansson *et al.* 2008; Halaihel *et al.* 2009; Sandell & Jacobson 2011), none has been sufficiently extensive or rigorous to fully validate a particular method according to standardized published procedures (Westgard 2008; OIE 2009). Comparisons frequently have been made using tissues from fish of unknown *R. salmoninarum* infection status, making it difficult to evaluate non-concordant results among assays to determine which results actually represent 'true positives' and 'true negatives.'

The study described herein was a bench-top comparison of seven immunological and molecular *R. salmoninarum* detection assays [direct smear FAT (smear DFAT) for tissue samples, direct membrane-filtration FAT (MF-FAT) for fluid samples, polyclonal ELISA, nested PCR (nPCR)

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and three real-time quantitative PCR (qPCR) assays], with a quantitative bacteriological culture method serving as the benchmark standard. Selection criteria for each non-culture assay included the following: (i) the protocol has been described in a peer-reviewed publication, (ii) a standardized protocol has been developed and tested, (iii) the appropriate reagents are commercially available, and (iv) the method is currently being used by state, federal or private fish health laboratories. When possible, assays tested were among those recommended for *R. salmoninarum* detection or confirmation in the American Fisheries Society-Fish Health Section Blue Book (AFS-FHS 2005) or the Office International des Épizooties Manual of Diagnostic Tests for Aquatic Animals (OIE 2003) or both. Analytical specificity, sensitivity and repeatability were evaluated for all seven of the non-culture assays, and comparisons of diagnostic specificity and sensitivity were made with a subset of the assays. For all evaluations, matrices (kidney tissue, ovarian fluid or saline) seeded with known (culture-confirmed) concentrations of bacteria, or fish of known *R. salmoninarum* infection status, were used. The bench-top comparison identified strengths and weaknesses of each diagnostic method tested.

Materials and methods

Bacterial isolates and culture conditions

We tested the specificity and sensitivity of the various detection methods by use of a panel of bacteria representing the target organism (*R. salmoninarum*), non-target bacterial species that are phylogenetically related to *R. salmoninarum* (Wiens *et al.* 2008), including *Arthrobacter* species and other Micrococcaceae, and a range of other Gram-positive and Gram-negative obligate or opportunistic bacterial fish pathogens. Seventeen bacterial species, all obtained from the American Type Culture Collection (ATCC), were included in the non-target species panel (Table 1). Eleven isolates of *R. salmoninarum* were obtained from various salmonid species and geographic locations (Table 2). Because three of the PCR assays tested detect sequences of the *msa* gene encoding a 57-kDa protein called p57 or major soluble antigen (MSA) that is reported to be a major virulence factor (Bruno 1988; Senson & Stevenson 1999; O'Farrell, Elliott & Landolt 2000; Rhodes, Coady & Deinhard 2004; Coady *et al.* 2006), several *R. salmoninarum* isolates known to have different copy numbers of the *msa* gene (Rhodes *et al.* 2004) were included, to investigate whether this characteristic might influence the sensitivity of an assay or the quantitative abilities of the PCR assays. In addition, an attenuated *R. salmoninarum* strain (MT239) with reduced cell-associated MSA (O'Farrell & Strom 1999; Senson & Stevenson 1999) and a strain with a point mutation in the *msa* gene (684) that increases the agglutination of salmonid leucocytes (Wiens, Pascho & Winton 2002) were tested.

Table 1 Panel of non-target bacteria used for analytical specificity testing

Bacterial species	ATCC number
Arthrobacter globiformis	8010
Arthrobacter protophormiae	19271
Kocuria (Micrococcus) varians	15306
Micrococcus luteus	4698
Mycobacterium marinum	927
Nocardia asteroides	19247
Carnobacterium maltaromaticum (piscicola)	35586
Leifsonia aquatica (Corynebacterium aquaticum)	14665
Aeromonas hydrophila	7966
Aeromonas salmonicida	33658
Edwardisiella tarda	15947
Flavobacterium johnsoniae (columnare)	43622
Flavobacterium pyschrophilum	49418
Pseudomonas fluorescens	13525
Vibrio ordalii	33509
Vibrio (Listonella) anguillarum	68554
Yersinia ruckeri	29473

ATCC, American Type Culture Collection.

Seed cultures of each non-target bacterium were prepared in the appropriate liquid medium and incubated at the recommended temperature, according to ATCC guidelines received with the cultures. The *R. salmoninarum* isolates were cultured for 8 days at 15 °C with constant stirring in KDM2 broth medium (Evelyn 1977) modified by the use of 0.05% (w/v) L-cysteine-HCl and 10% (v/v) foetal calf serum. Aliquots of the broth cultures (about 1 mL each) for all bacterial isolates were harvested and frozen at −80 °C for use as seed cultures. Concentrations of bacteria in the seed cultures were determined by serial dilution and plate counting on agar media, and purity of the cultures was verified by subcultures followed by Gram staining and wet mounts. The identity of each bacterial isolate was confirmed by sequencing a portion of the 16S rRNA gene (Clarridge 2004). To prepare stock cultures for the assays, a seed culture of a given bacterium was

Table 2 *Renibacterium salmoninarum* isolates used for analytical specificity and sensitivity testing

Isolate	Geographic origin (year)	Species	Source
ATCC 33209[a]	Leaburg Hatchery, Oregon USA (1974)	Spring Chinook salmon (*Oncorhynchus tshawytscha*)	ATCC
GL-64[a]	Lake Michigan USA (1990)	Fall Chinook salmon (*O. tschawytscha*)	R. A. Sonstegard (MU)
CHLM 91-02b	Little Manistee Weir, Michigan USA (2002)	Fall Chinook salmon	M. Faisal (MSU)
M05-44583-33K (abbreviated M05-33K)	Kettle Moraine Springs Hatchery, Wisconsin USA(2005)	Steelhead (*Oncorhynchus mykiss*)	H-M Hsu (WVDL)
M04-28852-35 (abbreviated M04-K35)	Besadny Spawning Facility, Wisconsin USA(2004)	Coho salmon (*Oncorhynchus kisutch*)	S. Marcquenski (WIDNR)
M05-86303-11 (abbreviated M05-BNT)	Wild Rose Hatchery, Wisconsin USA (2005)	Brown trout (*Salmo trutta*)	H-M Hsu (WVDL)
MMMvir[b]	Seattle, Washington USA (1999)	Sockeye salmon (*Oncorhynchus nerka*)	L.D. Rhodes (NWFSC)
Willamette[c]	Willamette Hatchery, Oregon USA (1993)	Spring Chinook salmon	L.D. Rhodes (NWFSC)
GR5	Montana USA (1997)	Arctic grayling (*Thymallus arcticus*)	E. MacConnell (USFWS)
MT 239	Aberdeen, Scotland (1988)	Atlantic salmon (*Salmo salar*)	J.G. Daly (UG)
684	Aurland Sognefjord, Norway	Brown trout	G.D. Wiens (USDA-ARS)

ATCC, American Type culture Collection, Manassas, Virginia USA; MU, McMaster University, Hamilton, Ontario, Canada; MSU, Michigan State University, East Lansing, Michigan USA; WVDL, Wisconsin Veterinary Diagnostic Laboratory, Madison, Wisconsin USA; WIDNR, Wisconsin Department of Natural Resources, Madison, Wisconsin USA; NWFSC, Northwest Fisheries Science Center, NOAA, Seattle, Washington USA; USFWS, U.S. Fish and Wildlife Service Bozeman Fish Technology Center, Bozeman, Montana USA; UG, University of Guelph, Guelph, Ontario, Canada; USDA-ARS, U.S. Department of Agriculture-Agricultural Research Service National Center for Cool and Coldwater Aquaculture, Kearneysville, West Virginia USA.

[a]Isolates with two *msa* gene copies (Rhodes *et al.* 2004).

[b]Isolate with three *msa* gene copies (Rhodes *et al.* 2004).

[c]Isolate with four *msa* gene copies (Rhodes *et al.* 2004).

grown in the appropriate broth medium, centrifuged at 5000 ***g*** for 20 min at 4 °C; the supernatant was discarded; and the pellet was resuspended to a standard concentration in the assigned diluent for each task. The concentration of live bacteria was determined by plate counts. The stock cultures were further diluted as necessary for a particular task.

Diagnostic assays

Culture. The bacteriological culture protocol of Jansson *et al.* (1996), modified from the method of Evelyn, Ketcheson & Prosperi-Porta (1981), was used for the detection and enumeration of viable *R. salmoninarum* in homogenized kidney tissue samples. Homogenized tissue samples were diluted at 10 mL g^{-1} of tissue in 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.1% (w/v) peptone (PBS-peptone). Diluted samples were mixed with a vortex mixer and then centrifuged at 2500 ***g*** for 20 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in PBS-peptone at a 1:1 ratio (w/v). Unless otherwise specified, a 10-μL volume of the resuspended pellet was spread onto the entire surface of each of three SKDM agar plates (Austin, Embley & Goodfellow 1983) with 1.5% (v/v) *R. salmoninarum*-conditioned medium (Evelyn, Prosperi-Porta & Ketcheson 1990), 10% (v/v) foetal bovine serum and 1.5% (w/v) agar included. Fluid samples (PBS or ovarian fluid) were spread directly onto the entire surface of three replicate SKDM agar plates (100 μL per plate) without prior processing. Additional serial 10-fold dilutions of samples were made in PBS-peptone before plating as appropriate to ensure countable numbers of colonies on plates. Culture plates were incubated at 15 °C for up to 12 weeks.

ELISA. A double-antibody sandwich polyclonal ELISA (ELISA II; Pascho *et al.* 1991) that measures the levels of a soluble antigen fraction of *R. salmoninarum* was used in this research. Unconjugated and horseradish peroxidase-conjugated polyclonal goat immunoglobulin to *R. salmoninarum* (Kirkegaard and Perry Laboratories) and a positive control antigen (Kirkegaard and Perry Laboratories) were used at the concentrations described by Pascho *et al.* (1991). Negative control samples included the appropriate matrix without *R. salmoninarum* added. Unless otherwise described, homogenized kidney

tissue samples were diluted 1:8 (w/v) in 0.01 M PBS, pH 7.4, containing 0.05% (by volume) Tween 20 (PBS-T20), and fluid samples were diluted with an equal volume of PBS-T20. Samples were then heated at 100 °C for 15 min and centrifuged at 10 000 ***g*** for 6 min at 4 °C before testing. Each sample was tested in duplicate, with 200 μL of sample supernatant added into each ELISA plate well. The negative–positive threshold absorbances were calculated by the method of Pascho *et al.* (1987) except where otherwise noted. Absorbance or optical density (OD) values between the negative–positive threshold and 0.199 were considered to have low antigen levels, values between 0.200 and 0.999 were considered to have moderate antigen levels, and values ≥ 1.000 were considered to have high antigen levels.

Direct smear FAT. Smears of homogenized kidney tissue for DFAT were made by dipping a fine-tipped calcium alginate swab (Fisher Scientific) into the tissue homogenate for a given sample and then spreading a thin film of the homogenate onto each well of a 2-well FAT slide (Erie Scientific; 8-mm diameter wells). Fixation and staining of the smears was carried out as described by Pascho *et al.* (1987) except that affinity-purified goat anti-*R. salmoninarum* immunoglobulin conjugated with fluorescein isothiocyanate (FITC) was obtained from Kirkegaard and Perry Laboratories. Before each use, the conjugate was diluted 1:40 (v/v) in 0.01 M PBS, pH 7.1, and filtered through a 0.2-μm filter. Stained smears were observed using epifluorescence microscopy at 1000× magnification. Fifty microscope fields were examined in each well in a non-overlapping grid pattern, for a total of 100 fields per slide. Only intact, labelled bacterial cells showing the correct size and morphology were counted. A positive control slide with smears of kidney homogenate from a known *R. salmoninarum*-positive fish was included with each set of slides stained.

Membrane-filtration FAT. The direct MF-FAT procedure of Elliott & McKibben (1997) was used for testing fluid samples only. The volume of each sample filtered was 500 μL, and a single filter was prepared for each sample. The same FITC-conjugated anti-*R. salmoninarum* immunoglobulin preparation used for kidney tissue DFAT was employed for MF-FAT. Filters were examined using epifluorescence microscopy at 1000× magnification, with 150 microscope fields examined per filter in a non-overlapping grid pattern. When necessary, serial 10-fold dilutions of samples were made to achieve countable numbers of bacteria on filters. Criteria for counting cells were the same as for smear DFAT. A positive control consisting of the appropriate matrix seeded with *R. salmoninarum* from an infected fish and a negative control consisting of reagents only were filtered and tested with each set of samples.

DNA extraction for PCR. The DNA was extracted from samples using a DNeasy tissue kit (Qiagen, Inc.), following the manufacturer's instructions for Gram-positive bacteria as described by Chase *et al.* (2006). A 50-μL volume of each fluid sample (in PBS or ovarian fluid matrix) was subjected to extraction. The weight of each sample in the kidney tissue matrix was recorded prior to DNA extraction; the target weight was about 25 mg. The DNA was eluted with 200 μL of buffer AE (Qiagen) for all tasks except repeatability testing, for which a 400-μL elution volume was used. Negative extraction controls containing reagents only were processed concurrently to verify that each extraction was free of contaminating DNA.

Nested PCR. This nPCR is designed to detect a 320-basepair (bp) region of the *R. salmoninarum msa* gene (Chase & Pascho 1998). The nPCR procedure described by Pascho, Chase & McKibben (1998) was used, with the following modifications: for the first round, 20 pM of each of the primers P3 and M21 was used (Table 3), and PCR conditions included 30 cycles of denaturing in a thermal cycler at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The second round reaction mixture included 20 pM of each of the primers P4 and M38 (Table 3), and DNA was amplified with the thermal cycler program described for the first round except with 20 cycles. The second-round PCR product for each sample was analysed using gel electrophoresis (one lane per sample) by the method of Pascho *et al.* (1998). A 5-μL volume of extracted DNA was used as template DNA for the first-round PCR, and 1 μL of first-round amplification product served as template DNA for the second-round PCR. Controls for each PCR run included the negative extraction control described above, a negative control with no template DNA, and a positive control consisting of DNA extracted from an *R. salmoninarum* culture.

Table 3 Primer and probe sequences used for PCR detection of *Renibacterium salmoninarum*. Note that some of the probes used for qPCR in this study utilized a minor groove binding (MGB) linker with a non-fluorescent quencher (NFQ) that differed from those used in the original publications

Target gene	Assay	Name	Sequence (5′–3′)	References
msa	nPCR	P3 (outer F)	AGCTTCGCAAGGTGAAGGG	Pascho *et al.* (1998)
		M21 (outer R)	GCAACAGGTTTATTTGCCGGG	
		P4 (inner F)	ATTCTTCCACTTCAACAGTACAAGG	
		M38 (inner R)	CATTATCGTTACACCCGAAACC	
msa	qPCR #1	RS1238 (F)	GTGACCAACACCCAGATATCCA	Chase *et al.* (2006)
		RS1307 (R)	TCGCCAGACCACCATTTACC	
		RS1262 NFQ probe[a]	6FAM-CACCAGATGGAGCAAC-MGB/NFQ	
msa	qPCR #2	250F (F)	CAACAGGGTGGTTATTCTGCTTTC	Powell *et al.* (2005)
		344R (R)	CTATAAGAGCCACCAGCTGCAA	
		300T probe	6FAM-CTCCAGCGCCGCAGGAGGAC-TAMRA	
abc	qPCR #3	ABCtransfor2	CTAAACGATTTCCCGGTCAA	Rhodes *et al.* (2006)
		ABCtransrev2	GATTTTGCCTGCTGGTATTTCC	
		ABC NFQ probe[b]	6FAM-AAGCGCCAGCAGTCGACGGC- MGB/NFQ	

[a]In the original publication (Chase *et al.* 2006), the 3′ end of the RS1262 probe was labelled with TAMRA.
[b]In the original publication (Rhodes *et al.* 2006), the 3′ end of the ABCtrans probe was labelled with TAMRA.

Real-time quantitative PCR #1. This qPCR detects a 69-bp region of the *R. salmoninarum msa* gene (Chase *et al.* 2006). The primer and probe sequences (Table 3) were selected from a different region of the *msa* gene than the primers used for the nPCR of Pascho *et al.* (1998), to avoid the possibility of contamination from amplified nPCR products in the laboratory. The DNA extraction was carried out as previously described. The qPCR were performed and analysed using an ABI 7900HT sequence detection system (Applied Biosystems) according to the method of Chase *et al.* (2006) with the following changes: the probe was modified to contain a minor groove binder (MGB) linker and a non-fluorescent quencher (Applied Biosystems) (Table 3), and the PCR reaction mixture included 6 μL of 2× TaqMan® Universal Master Mix (Applied Biosystems), 900 nM of each *R. salmoninarum*-specific primer, 250 nM of *R. salmoninarum*-specific probe, and 5 μL of template DNA in a total volume of 12 μL. For the quantification of the amount of *R. salmoninarum* in a sample, a standard curve of serially diluted *R. salmoninarum* DNA was used representing a range of 5×10^5 cells per reaction to 5 cells per reaction (Chase *et al.* 2006). Standard curve analyses were carried out in triplicate, and the samples were run in duplicate. Samples were considered positive for *R. salmoninarum* DNA if both replicates showed cycle quantification (C_q) values less than 38, representing the theoretical lower limit of consistent detection (≥ 5 *R. salmoninarum* cells per reaction; Chase *et al.* 2006). (Note: C_q values are called cycle threshold or C_T values in Applied Biosystems Inc. Sequence Detection Software.)

Real-time quantitative PCR #2. This qPCR detects a 95-bp sequence of the *R. salmoninarum msa* gene (Powell *et al.* 2005). The primer and probe sequences designed for this qPCR are located on the *msa* gene between the first-round forward and reverse primer sequences (P3 and M21) previously selected for the nPCR of Pascho *et al.* (1998) (Table 3). The qPCR probe is labelled with the quencher dye TAMRA on the 3′ end (Table 3). The qPCR #2 assay was performed as described by Powell *et al.* (2005), and controls and standards were the same as for qPCR #1.

Real-time quantitative PCR #3. This qPCR detects a 70-bp region of the *R. salmoninarum* abc transporter permease (*abc*) gene, a single-copy gene (Rhodes *et al.* 2006). The primer and probe sequences were the same as described by Rhodes *et al.* (2006) except that the probe was modified to contain an MGB linker and a non-fluorescent quencher (Table 3). The procedure of Rhodes *et al.* (2006) was followed for qPCR #3, but because this published protocol did not specify a PCR master mix, we used the TaqMan® Universal Master Mix (Applied Biosystems) specified for qPCR #1 (Chase *et al.* 2006) and qPCR #2 (Powell *et al.* 2005). Controls and standards were the same as described for qPCR #1.

Assay matrices

Separate suspensions of each bacterium were prepared in each of three matrices: (i) 0.01 M PBS, pH 7.4 (the reference matrix, free of potential effects of the physical and chemical properties of a fish tissue or fluid sample), (ii) homogenized salmonid kidney tissue, and (iii) salmonid ovarian fluid.

Kidney tissue from Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), spawning at the Soos Creek Hatchery (Green River Complex, Washington State Department of Fish and Wildlife, Auburn, Washington USA) was collected to prepare the homogenate to be used as the kidney tissue matrix. Entire kidneys were collected from 159 adult male and female fish in September and October 2003 and October 2004. The kidney tissue from each fish was homogenized with a stomacher and a print roller before testing.

Cultures were made on KDM2 agar medium, incubated at 15 °C for a minimum of 81 days, and examined three times for growth of colonies resembling *R. salmoninarum*. Suspicious colonies were tested by DFAT for specific staining and morphological features as described above. All kidney samples were also tested by polyclonal antibody ELISA by the procedure described above, with the exception that the kidney homogenate was diluted 1:4 (w/v) in PBS-T20 before processing and testing by ELISA. Tissues that tested negative for *R. salmoninarum* by culture and ELISA were further analysed by nPCR as previously described. Only samples testing negative for *R. salmoninarum* by all the assay procedures were included in the pooled tissue matrix.

Ovarian fluid was also collected from 89 spawning female Soos Creek Hatchery Chinook salmon in 2003 and 2004, to be tested for inclusion in the ovarian fluid matrix. Samples were cultured on KDM2 medium and tested by the ELISA as previously described. Ovarian fluid samples that tested negative for *R. salmoninarum* by culture and ELISA were processed and tested by nPCR. Selection criteria for the inclusion of a given ovarian fluid sample in the pool for the matrix were the same as for the kidney tissue.

The kidney tissue matrix was prepared by pooling the *R. salmoninarum*-negative kidney tissue and mixing it in a large food processor (10 kidneys at a time), followed by mixing all samples together in a large bowl. The ovarian fluid matrix was prepared by pooling the *R. salmoninarum*-negative fluid in a large bottle and mixing it by shaking. The kidney and ovarian fluid matrices were stored as aliquots in tubes at −80 °C.

In an initial experiment with seeded kidney tissue and ovarian fluid, bacteriological culture did not detect *R. salmoninarum* at concentrations below $\log_{10}$ 5.8 colony forming units (CFU) mL^{-1}. The effectiveness of culture was reduced by the presence of contaminating organisms in the kidney tissue and ovarian fluid, which likely obscured the presence of *R. salmoninarum* on the culture plates, or inhibited the growth of the target bacterium, or both. The growth of contaminants occurred despite the use of SKDM medium containing antibacterial and antifungal agents. Pasteurization was found to be effective for eliminating contaminants that interfered with culture results, but did not significantly change results for the non-culture assays in preliminary experiments (data not shown). Therefore, testing of the non-culture detection methods in comparison with culture for the detection of the various *R. salmoninarum* isolates was continued with the use of pasteurized kidney tissue and ovarian fluid.

For pasteurization, kidney or ovarian fluid samples were thawed and placed into a sterile glass beaker containing a stir bar. This sample beaker was placed into a larger glass beaker containing water maintained at 65 °C, using a hot plate to heat the water. A thermometer was placed into the beaker containing the sample, and the temperature was monitored until it reached 63 °C, while stirring. The sample was then maintained at a temperature between 63 and 65 °C for 30 min. At the end of the incubation, the tissue or ovarian fluid sample was removed from heat and placed into an ice bath to rapidly cool the sample to 4 °C. Aliquots of tissue samples were made in a sterile hood and placed at −80 °C for long-term storage or left at 4 °C for immediate use. The kidney tissue and ovarian fluid matrices were thawed and seeded with *R. salmoninarum* according to procedures described in each task.

Analytical specificity

The goal of this task was to determine whether a given non-culture method detected all 11 isolates of *R. salmoninarum* tested (Table 2), while failing to produce a positive result with samples containing any of the non-target bacteria (Table 1). For testing the specificity of *R. salmoninarum* non-

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culture detection methods, stock cultures were diluted as appropriate in PBS, pH 7.4, containing 0.01% (w/v) of thimerosal to make final concentrations of about $\log_{10}$ 8, $\log_{10}$ 7 and $\log_{10}$ 6 bacteria mL^{-1}, and frozen in aliquots at −80 °C. Three bacterial isolates, *Mycobacterium marinum*, *Nocardia asteroids* and *Vibrio ordalii*, showed strong auto-aggregation in culture, which prevented accurate enumeration of individual bacteria. These bacteria were diluted by weight to make final concentrations of 5, 2 and 1 mg mL^{-1}. The MF-FAT, ELISA, nPCR, qPCR #1, qPCR #2 and qPCR #3 assays were performed as previously described.

Analytical sensitivity

This task was designed to determine the lowest concentration of each *R. salmoninarum* isolate (Table 2) detectable by each non-culture method in each of the three matrices. Culture, harvesting and preparation of stock suspensions were carried out as previously described. Serial 10-fold dilutions of stock suspensions were then made in PBS (for seeding the PBS matrix) or PBS-peptone (to seed the kidney tissue and ovarian fluid matrices). Seeding of kidney tissue was achieved by mixing a 100-μL aliquot of a given bacterial dilution in a tube with 1.5 g of the pooled kidney homogenate, and seeding of ovarian fluid was accomplished by mixing a 200 μL aliquot of a given bacterial dilution with 2.0 mL of pooled ovarian fluid. The target range of seeding concentrations was about $\log_{10}$ 1 to $\log_{10}$ 8 *R. salmoninarum* cells per sample in each matrix. The serial dilutions of each isolate preparation by culture and each non-culture method were tested as previously described.

Repeatability

The goal of this task was to measure the repeatability both within and between analyses to estimate the variability inherent to each detection method when tested with *R. salmoninarum* isolate GL-64 (Table 2) suspended at three different concentrations in each of the three matrices. Target *R. salmoninarum* concentrations in the matrices were the following: $\log_{10}$ 6.7 CFU mL^{-1} or g^{-1} (high), $\log_{10}$ 5.4 CFU mL^{-1} or g^{-1} (medium), and $\log_{10}$ 4.0 CFU mL^{-1} or g^{-1} (low). The kidney tissue and ovarian fluid were pasteurized before seeding with *R. salmoninarum*. For each detection method, an internal standard was included in each run to ensure that experimental error introduced because of deteriorating reagents or other technical difficulties did not affect the measurements.

Within-run repeatability. The within-run repeatability of the ELISA and qPCR was estimated by performing 30 replicate determinations (in the same run) on each of 10 samples at each concentration. Because of the increased labour involved in smear DFAT, MF-FAT and nPCR assay performance, within-run repeatability of these assays was estimated by performing 10 replicate determinations (in the same run) on each of five samples at each concentration. For PCR, the DNA extractions for all replicates of a given sample were carried out at the same time.

To prepare the samples in the PBS matrix at a given *R. salmoninarum* concentration, 500 mL of PBS was seeded with the appropriate amount of *R. salmoninarum*, which had been prepared, enumerated and stored at −80° C as previously described. The seeded PBS was stirred on ice for 5–10 min, and then, 1 mL was removed for serial 10-fold dilution to prepare culture plates. The seeded PBS was divided into 10 aliquots of 48 mL each. Each of these aliquots became the individual samples, which were then divided into 30 replicates for each of the assays and frozen at −80 °C until further processing and testing by a given assay. The assays were performed as previously described. For the ovarian fluid matrix, *R. salmoninarum* seeding procedures were similar to those used for seeding the PBS matrix. For within-run repeatability testing in the kidney matrix, 100 g of kidney tissue was seeded with *R. salmoninarum* at each of three concentrations as described above, except that the seeded tissue was stirred for at least 16 h (overnight) on a stir plate at 4 °C to ensure thorough mixing.

Between-run repeatability. To measure the between-run repeatability of each assay, the 10 samples at each concentration were tested in 30 separate runs on different days (ELISA and qPCR), or five samples at each concentration were tested in 10 separate runs (smear DFAT, MF-FAT and nPCR). Processing procedures for samples seeded in the three matrices were similar to those for within-run repeatability testing. However, because the DNA extraction protocols limited the

amount of sample extracted to 50 μL for fluid samples and 25 mg for tissue homogenates, extractions were performed on several aliquots of each sample, and extracted DNA was pooled to yield a total volume of 2 mL of extracted DNA per sample. The extracted DNA was divided into thirty 60-μL aliquots for 30 separate runs and stored at 4 °C until testing.

Diagnostic sensitivity and specificity

The aim of this task was to determine for each assay the proportion of known *R. salmoninarum*-positive reference fish that tested positive (diagnostic sensitivity), and the proportion of known *R. salmoninarum*-negative reference fish that tested negative (diagnostic specificity). Broodstock screening and a laboratory *R. salmoninarum* challenge were used to create reference animals for preliminary estimates of diagnostic sensitivity and specificity for the assays.

Obtaining SPF Chinook salmon. Screening of spawning fish for *R. salmoninarum* by ELISA is effective in minimizing vertical transmission of the bacterium (Pascho *et al.* 1991; Munson *et al.* 2010). Although this method does not guarantee that all fish are completely specific pathogen-free (SPF), it has been used successfully to create groups of SPF fish for laboratory studies (Coady *et al.* 2006; Purcell *et al.* 2008; Metzger *et al.* 2010). Spawning fall Chinook salmon to provide SPF progeny for this research were obtained from Strawberry Creek, Wisconsin in October 2004. Kidney tissue samples from female and male fish of 42 mating pairs and ovarian fluid samples from the female fish were tested for *R. salmoninarum* by ELISA as previously described. Ovarian fluid samples that showed ELISA values at or below the negative–positive threshold were also tested by MF-FAT. Five families were selected on the basis of negative kidney tissue results by ELISA testing, negative or borderline positive ovarian fluid results by ELISA testing, and negative or borderline positive ovarian fluid results by MF-FAT testing (≤ 1 bacteria in 150 microscope fields). In November 2004, eyed eggs from the selected families were transferred from Wild Rose Hatchery, Wisconsin to the Western Fisheries Research Center in Seattle, Washington. The fish were hatched and reared in sand-filtered, UV-treated Lake Washington water for 2 years prior to challenge.

Challenge of SPF Chinook salmon. Cultures of *R. salmoninarum* isolate GL-64 were prepared for challenge according to the procedure of McKibben & Pascho (1999). For the challenge, the bacteria were resuspended in PBS-peptone, and the concentration of *R. salmoninarum* in the final suspension was determined by MF-FAT and plate counts. Juvenile Chinook salmon (average weight 61.9 g) were challenged with *R. salmoninarum* by the injection protocol of McKibben & Pascho (1999). A group of 150 Chinook salmon was acclimated over a 1-week period to a water temperature of 15 °C, and then, each fish was injected intraperitoneally with $\log_{10}$ 6.05 *R. salmoninarum*. A group of 100 control fish in a separate tank was acclimated but was not injected. Both groups were held at 15 °C for 15 days prior to sampling. The *R. salmoninarum* challenge dose and sampling time were selected on the basis of results of previous challenges (e.g., Purcell *et al.* 2008; Metzger *et al.* 2010), which indicated that the infection would likely be progressing at the time of sampling, but that most fish would probably have low to moderate infection levels.

At sampling, fish were removed from the tank, euthanized with an overdose of buffered tricaine methanesulfonate (MS-222, Argent Chemical Laboratories), and the entire kidney was removed. To minimize cross-contamination, new tools and gloves were used to sample each individual fish. Following the removal of the kidney, the tissue was placed into a stomacher bag and a photographic print roller was used to homogenize the entire kidney. Small portions of the homogenized kidney were then distributed into preweighed labelled tubes for PCR, ELISA and culture. A Dacron swab was used to spread the kidney tissue onto two wells of a glass slide for DFAT. All sample tubes were randomized and coded so that the tissue would be tested without bias. Culture and the non-culture *R. salmoninarum* assays were performed as previously described, with some modifications. Serial 10-fold dilutions (to 10^{-2}) in PBS-peptone were made from the kidney homogenate samples from *R. salmoninarum*-injected fish to achieve quantifiable numbers of bacteria on culture plates, with a single SKDM plate inoculated from the original homogenate and at each dilution. The identity of colonies showing morphological characteristics consistent with *R. salmoninarum* in culture was confirmed by DFAT. Among the PCR assays, only nPCR

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and qPCR #1 could be performed owing to a loss of samples during freezer storage.

Statistical analysis

Statistical analyses were performed with InStat 3, Graph Pad Software, Inc. and IBM SPSS V.18 (IBM Inc.). For comparisons of analytical sensitivity among assays, log-transformed *R. salmoninarum* concentration data were first tested for normality by the Kolmogorov–Smirnov method. Because at least one data set in each comparison failed the normality test ($P < 0.05$), the nonparametric Kruskal–Wallis test (single factor analysis of variance by ranks) was used for comparisons among assays of the lowest *R. salmoninarum* concentrations detected in a matrix. Dunn's multiple comparison test was applied when a significant result ($P < 0.05$) was observed using the Kruskal–Wallis test.

Linear regression analysis was performed on log-transformed data to investigate the relation between *R. salmoninarum* seeding concentrations and quantity estimates determined by culture, MF-FAT and the qPCR assays in a given matrix. Analysis of variance was used to test the significance of a regression slope and to test for deviations from linearity. Pearson's correlation analysis of log-transformed data was carried out to evaluate correlations between *R. salmoninarum* quantity estimates obtained by these assays.

Diagnostic sensitivity and specificity of the assays, likelihood ratios (LRs) for positive and negative test results, and 95% confidence intervals (CI) were calculated as described by Thrusfield (2005). A LR more than 10 for a positive test was considered strong evidence that the test result came from an *R. salmoninarum*-infected fish rather than an uninfected fish, and a LR <0.1 for a negative test was considered strong evidence that the test result came from an uninfected rather than from an infected fish (Akobeng 2006). For the ELISA data, a receiver operating characteristic (ROC) curve was used to generate the data for true-positive and false-positive detection rates at several negative–positive threshold ELISA OD values (Thrusfield 2005). As a measure of diagnostic test accuracy, the diagnostic odds ratio (DOR) was calculated for each assay (and for various ELISA OD cut-off values) to determine the ratio of the odds of *R. salmoninarum* infection in positive test results over the odds of infection in negative test results (Caraguel *et al.* 2011). Concordance analysis was performed to evaluate the proportion of all test results (positive and negative) on which two different assays agreed, and κ statistic calculations were made to express the proportion of potential agreement beyond chance (Smith 2006). Spearman's rank correlation analysis was used to test for the correlations between *R. salmoninarum* levels estimated by ELISA, culture, smear DFAT and qPCR.

Results

Analytical specificity

Each of the non-culture assays detected all of the 11 *R. salmoninarum* isolates suspended in PBS. Specificity testing with each of the non-target species showed no apparent cross-reactivity in any of the assays except the polyclonal ELISA. Testing of bacteria suspended in PBS yielded borderline positive ELISA reactions with two Gram-negative fish pathogens, *Pseudomonas fluorescens* and *Vibrio ordalii*, at the highest concentration of bacteria only ($\log_{10}$ 8.0 bacteria mL^{-1}) when the negative–positive threshold value was calculated by the method of Pascho *et al.* (1987) (mean OD value of the negative control samples +2 SD). For both *P. fluorescens* and *V. ordalii*, the ELISA OD values obtained for the positive samples (0.063 and 0.064, respectively) were near the negative–positive cut-off value (first positive = 0.063). Retesting of these samples gave an OD value below the negative–positive cut-off (first positive = 0.071) for *P. fluorescens* (0.069), but the *V. ordalii* sample still tested borderline positive (0.071). Testing by qPCR #2 yielded apparent borderline positive results with five of the non-target bacterial species, but further testing revealed that the initial results were caused by low-level contamination.

Analytical sensitivity

In tests with 11 *R. salmoninarum* isolates, culture detected the lowest mean bacterial concentrations in all three matrices (Table 4). Testing of *R. salmoninarum* suspended in the PBS matrix yielded mean limits of detection (±SD) ranging from $\log_{10}$ 0.7 (±0.4) bacteria mL^{-1} for culture to $\log_{10}$ 5.4 (±0.9) bacteria mL^{-1} for ELISA. For the PBS matrix, the analytical sensitivity of culture and MF-FAT was significantly greater ($P < 0.05$)

than the analytical sensitivity of ELISA, nPCR and the three qPCRs. The lowest mean *R. salmoninarum* concentrations (±SD) detected in the ovarian fluid matrix ranged from $\log_{10}$ 1.0 (±0.4) bacteria mL^{-1} for culture to $\log_{10}$ 4.7 (±1.2) bacteria mL^{-1} for ELISA. For this matrix, the analytical sensitivity of culture was significantly higher ($P < 0.05$) than that of ELISA, nPCR, qPCR #1 and qPCR #3, and the analytical sensitivity of MF-FAT was significantly greater ($P < 0.05$) than that of ELISA and nPCR. Mean *R. salmoninarum* detection limits (±SD) in the kidney matrix ranged from $\log_{10}$ 1.9 (±0.5) bacteria g^{-1} for culture to $\log_{10}$ 5.9 (±1.0) bacteria g^{-1} for qPCR #3. Six of the 11 *R. salmoninarum* isolates were not detected by qPCR #3 in the homogenized kidney tissue at any seeding level, suggesting that inhibition of qPCR #3 occurred in this matrix. For the kidney matrix, the analytical sensitivity of culture was significantly greater ($P < 0.05$) than the analytical sensitivity of smear DFAT, ELISA and qPCR #3, and the analytical sensitivity of qPCR #2 was significantly higher ($P < 0.05$) than that of qPCR #3.

The percentage of the 11 *R. salmoninarum* isolates detected by each of the quantitative assays (culture, MF-FAT for PBS and ovarian fluid, and the three qPCRs) is shown for the PBS, ovarian fluid and kidney matrices in Figs 1–3, respectively. Culture and MF-FAT detected 100% of the isolates at all seeding concentrations above $\log_{10}$ 1 bacteria mL^{-1}. The data for the qPCR assays indicated the most consistent detection of *R. salmoninarum* at seeding concentrations $\geq \log_{10}$ 4 bacteria mL^{-1} (PBS and kidney, Figs 1 & 3) or $\geq \log_{10}$ 3 (ovarian fluid, Fig. 2), with the highest PCR efficiency for *R. salmoninarum* detection in the ovarian fluid matrix. Apparent inhibition of qPCR #3 was evident in the kidney matrix (Fig. 3). In tests with each PCR assay, DNA amplification was frequently observed for a given *R. salmoninarum* isolate at a low seeding concentration ($\log_{10}$ 0–$\log_{10}$ 2 bacteria g^{-1} or mL^{-1} as determined by culture) without further (and consistent) amplification occurring until the seeding concentration was at least 2 $\log_{10}$ higher (data not shown). This 'skip amplification' at low seeding concentrations likely represented the detection of randomly distributed *R. salmoninarum* DNA copies and was not included in calculations to determine the limits of consistent *R. salmoninarum* detection by an assay. (Similar 'skip detection' of *R. salmoninarum* antigen observed for ELISA at low seeding concentrations was also eliminated from calculations of analytical sensitivity for that assay as well; data not shown.)

Culture and MF-FAT results showed approximate $\log_{10}$ changes in *R. salmoninarum* quantity estimates (Figs 1–3) at each successive 10-fold change in seeding concentration in a matrix as indicated by values approaching 1.0 for the linear regression slopes and relatively high coefficients of determination ($r^2 \geq 0.900$) (Table 5). Within the operating ranges of the qPCR assays, all showed linear regression slopes significantly different from 0 ($P \leq 0.001$) but $r^2 \leq 0.824$, and ANOVA testing did not reveal significant deviations from linearity ($P \geq 0.20$) (Table 5). Below the seeding concentration range for consistent qPCR detection of *R. salmoninarum* in a given matrix, quantity estimates by qPCR were unreliable (Figs 1–3).

Renibacterium salmoninarum quantity estimates were significantly correlated ($P < 0.05$) between culture and the other quantitative assays. The highest correlations (Pearson's r) of quantity estimates were observed between culture and MF-FAT for both PBS and ovarian fluid ($r > 0.99$, $P < 0.0001$). Correlations of quantity estimates between culture and qPCR #2 for PBS ($r = 0.92$, $P = 0.01$), ovarian fluid ($r = 0.97$ $P = 0.001$), and kidney ($r = 0.95$, $P = 0.004$) were higher than the equivalent correlations between culture and the other two qPCRs. Correlations of

Table 4 Analytical sensitivity of assays for detection of 11 *Renibacterium salmoninarum* isolates seeded into three matrices. The detection limits are based on concentrations of viable bacteria determined by culture for each isolate at each seeding level for a given matrix. Six of the isolates seeded into kidney tissue were not detected by qPCR #3 at any seeding concentration

	$\log_{10}$ mean detection limit mL^{-1} or g^{-1} ± SD		
Assay	PBS	Ovarian fluid	Kidney
Culture	$0.7^{a} \pm 0.4$	$1.0^{a} \pm 0.4$	$1.9^{a} \pm 0.5$
MF-FAT	$1.1^{a} \pm 0.9$	$1.7^{ab} \pm 0.7$	–
Smear FAT	–	–	$4.6^{bc} \pm 2.2$
ELISA	$5.4^{b} \pm 0.9$	$4.7^{c} \pm 1.2$	$4.8^{bc} \pm 0.7$
nPCR	$3.8^{b} \pm 1.4$	$3.8^{c} \pm 1.5$	$3.6^{abc} \pm 2.2$
qPCR #1	$4.4^{b} \pm 1.2$	$2.9^{bc} \pm 1.0$	$2.9^{abc} \pm 2.4$
qPCR #2	$4.1^{b} \pm 1.4$	$2.5^{abc} \pm 0.7$	$2.4^{ab} \pm 2.1$
qPCR #3	$4.6^{b} \pm 1.0$	$3.1^{bc} \pm 1.0$	$5.9^{c} \pm 1.0$

Within each column, mean values not sharing a common superscript are significantly different ($P < 0.05$).

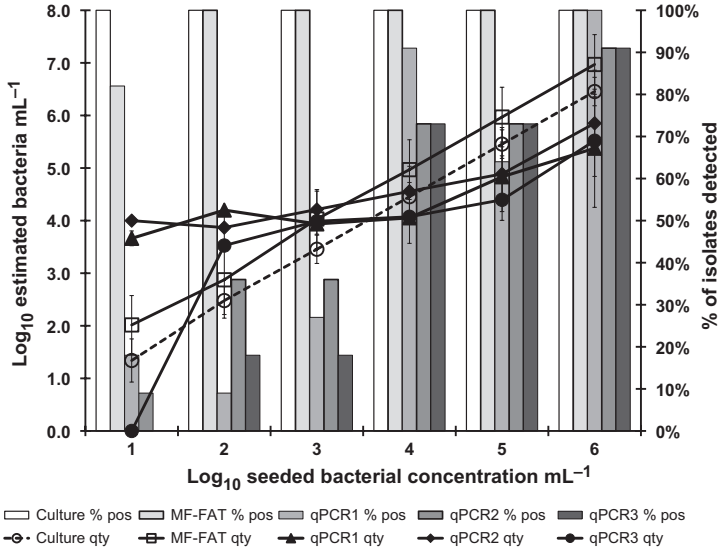

Figure 1 Percentage of 11 *Renibacterium salmoninarum* isolates detected (bars) by each quantitative assay (culture, MF-FAT, qPCR #1, qPCR #2, and qPCR #3) at each seeding concentration, and quantity estimates ($\log_{10}$ cells mL^{-1}) by each of these assays (lines) at each seeding concentration in the phosphate-buffered saline (PBS) matrix.

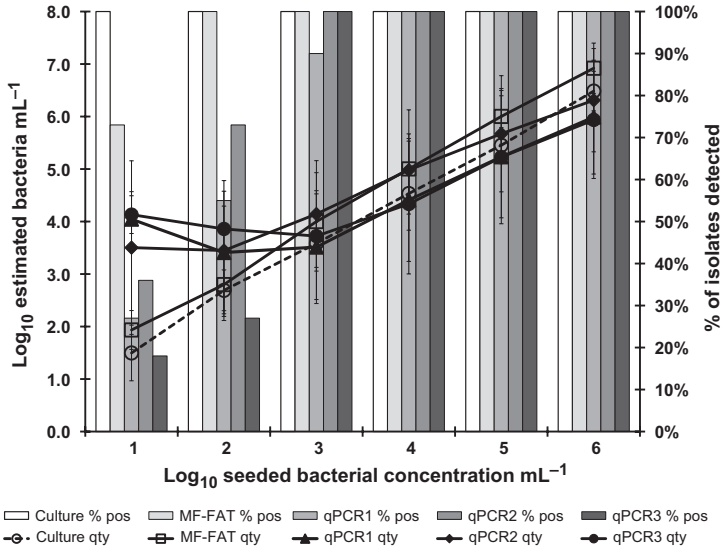

Figure 2 Percentage of 11 *Renibacterium salmoninarum* isolates detected (bars) by each quantitative assay (culture, MF-FAT, qPCR #1, qPCR #2, and qPCR #3) at each seeding concentration, and quantity estimates ($\log_{10}$ cells mL^{-1}) by each of these assays (lines) at each seeding concentration in the ovarian fluid matrix. The ovarian fluid was pasteurized before seeding with *R. salmoninarum*.

quantity estimates between culture and qPCR # 1 or qPCR #3 were similar for PBS ($r = 0.89$, $P = 0.02$ for qPCR #1; $r = 0.87$, $P = 0.02$ for qPCR #3) and ovarian fluid ($r = 0.83$, $P = 0.04$ for both assays). No correlation analysis was carried out for quantity estimates by culture and qPCR #3 in kidney tissue because of the low *R. salmoninarum* detection rates for qPCR #3 in this matrix, but the correlation of quantity estimates between culture and qPCR #1 was similar to that observed for the other matrices ($r = 0.86$, $P = 0.03$). Mean *R. salmoninarum* counts estimated by MF-FAT were generally higher than those estimated by culture (Figs 1 & 2), and mean *R. salmoninarum* counts estimated by qPCR #2 were generally higher than those estimated by qPCR #1 or qPCR #3 (Figs 1–3).

Repeatability

For the comparison of repeatability among quantitative and non-quantitative *R. salmoninarum* assays, the mean per cent positive results (*R. salmoninarum* detections), standard deviation (SD) and coefficient of variation (CV) were calculated by the method of Elder *et al.* (1997) for within-

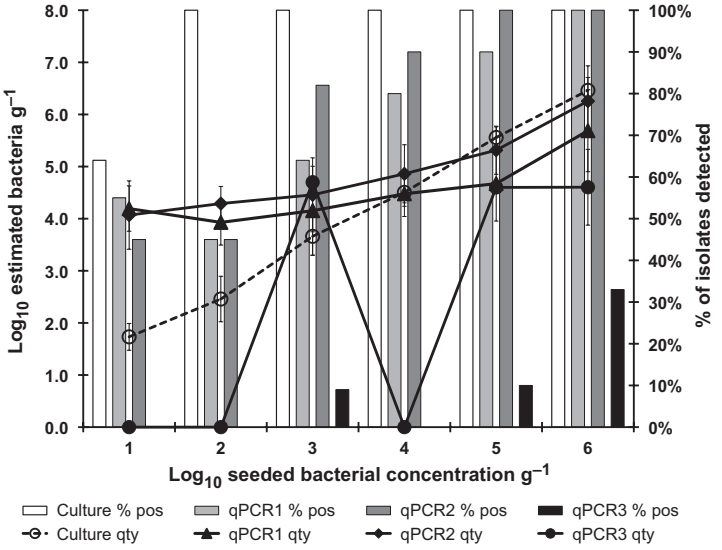

Figure 3 Percentage of 11 *Renibacterium salmoninarum* isolates detected (bars) by each quantitative assay (culture, qPCR #1, qPCR #2, and qPCR #3) at each seeding concentration, and quantity estimates (log_{10} cells g^{-1}) by each of these assays (lines) at each seeding concentration in the kidney tissue matrix. The homogenized kidney tissue was pasteurized before seeding with *R. salmoninarum*.

Table 5 Parameters determined by linear regression analysis of the relation between log_{10} *Renibacterium salmoninarum* seeding concentrations and log_{10} *R. salmoninarum* quantity estimates by culture, MF-FAT and qPCR in the seeded PBS, ovarian fluid and kidney tissue matrices. Data for the analyses were selected to encompass the operating range for each assay as determined by analytical sensitivity testing (log_{10} 1–6 seeding concentration range for culture and MF-FAT, log_{10} 4–6 seeding concentration range for the qPCRs in the PBS and kidney tissue matrices, and log_{10} 3–6 seeding concentration range for the qPCRs in the ovarian fluid matrix)

Matrix and assay	Slope	Y-intercept	r^2	Significance of regression (P)[a]	ANOVA for linearity (P)[b]
PBS					
Culture	1.014	0.390	0.973	<0.0001	0.89
MF-FAT	1.001	0.965	0.900	<0.0001	0.98
qPCR #1	0.660	1.444	0.344	0.001	0.75
qPCR #2	0.652	1.851	0.321	0.002	0.37
qPCR #3	0.793	1.031	0.430	0.0002	0.20
Ovarian fluid					
Culture	0.977	0.623	0.940	<0.0001	0.81
MF-FAT	1.018	0.881	0.918	<0.0001	0.89
qPCR #1	0.824	1.079	0.416	<0.0001	0.98
qPCR #2	0.720	2.039	0.382	<0.0001	0.96
qPCR #3	0.754	1.415	0.335	<0.0001	0.96
Kidney					
Culture	0.971	0.658	0.962	<0.0001	0.38
qPCR #1	0.490	2.537	0.371	0.0002	0.41
qPCR #2	0.583	2.593	0.544	<0.0001	0.37

[a]Null hypothesis: slope = 0.
[b]Null hypothesis: population regression is linear.

run and between-run testing of each assay at each *R. salmoninarum* concentration in a given matrix (Tables 6–8). For each assay, the variability was highest at concentrations near or below the threshold for consistent *R. salmoninarum* detection determined by analytical sensitivity testing (see Table 4). At seeding concentrations exceeding the threshold levels for a given assay, the *R. salmoninarum* detection rate generally approached 100% and the per cent CV approached 0. The lowest seeding concentrations in PBS and ovarian fluid (log_{10} 3.9–4.1) were higher than the detection threshold determined for MF-FAT during analytical sensitivity testing (log_{10} 1.1–1.7), and *R. salmoninarum* detection rates were 100% for this assay at all the concentrations tested (Tables 6 & 7). The mean *R. salmoninarum* detection rates for nPCR were 88–100%, and CVs were low

Table 6 Within-run and between-run repeatability of positive assay results for detection of *Renibacterium salmoninarum* isolate GL-64 seeded into phosphate buffered saline (PBS) at three concentrations

Repeatability test	*R. salmoninarum* assay					
	Mean % positive results ± SD (% CV[a])					
R. salmoninarum culture concentration $\log_{10}$ CFU mL^{-1}	MF-FAT[b]	ELISA[c]	nPCR[b]	qPCR #1[c]	qPCR #2[c]	qPCR #3[c]
Within-run						
3.9	100 (0)	15 ± 33 (220)	98 ± 5 (5)	84 ± 30 (36)	90 ± 25 (28)	21 ± 30 (143)
5.3	ND	77 ± 34 (44)	100[d] (0)	98 ± 5 (5)	97 ± 3 (3)	25 ± 28 (112)
6.8	ND	100 (0)	100[d] (0)	100 (0)	98 ± 2 (2)	36 ± 34 (94)
Between-run						
4.0	100 (0)	14 ± 7 (50)	100 (0)	87 ± 10 (11)	95 ± 3 (3)	42 ± 11 (26)
5.6	ND	76 ± 4 (5)	ND	100 (0)	98 ± 2 (2)	76 ± 10 (13)
6.6	ND	100 (0)	ND	100 (0)	100 (0)	83 ± 5 (6)

ND, not done.

[a]Per cent coefficient of variation (% CV) = (SD/mean) × 100.

[b]Within-run: 10 replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated five times. Between-run: five replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated 10 times (10 separate runs).

[c]Within-run: thirty replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated 10 times. Between-run: 10 replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated 30 times (30 separate runs).

[d]10 replicate samples at this concentration tested once rather than five times by nPCR.

(0–6%) at concentrations tested near the detection threshold in each matrix. For both MF-FAT and nPCR, the number of replicates or runs tested (10) was less than the number (30) tested for the other assays. Repeatability analysis could not be accomplished with qPCR #3 in the kidney matrix because of low detection rates (Table 8), and an unexpectedly low mean *R. salmoninarum* detection rate (36%) and high CV (94%) were observed for this assay during within-run repeatability testing in the PBS matrix at the highest seeding level ($\log_{10}$ 6.8 bacteria mL^{-1}) (Table 6).

Diagnostic sensitivity and specificity

Preliminary estimates and 95% confidence intervals (95% CI) for diagnostic sensitivity and specificity of assays for *R. salmoninarum* detection in kidney tissue were obtained using experimentally infected and uninfected juvenile Chinook salmon as *R. salmoninarum*-positive and *R. salmoninarum*-negative reference animals, respectively (Tables 9 & 10). The highest numbers of *R. salmoninarum*-injected fish tested positive by smear DFAT (Table 9) and ELISA (Table 10), followed by culture, qPCR #1, and nPCR (Table 9). Estimated diagnostic sensitivity (true-positive detection rate) ranged from 20% for nPCR to 76% for smear DFAT (Table 9). For ELISA, diagnostic sensitivity estimates ranged from 70% to 99% depending on the negative–positive cut-off value used (Table 10). Some uninjected fish tested positive for *R. salmoninarum* by smear DFAT and nPCR (Table 9) and by ELISA at lower cut-off OD values (Table 10). Estimates of diagnostic specificity (true-negative detection rate) ranged from 85% for smear DFAT to 100% for culture and qPCR #1 (Table 9). The estimated diagnostic specificity of ELISA ranged from 60% at a cut-off OD value of 0.064 to 100% at cut-off OD values ≥ 0.072 (Table 10).

The LRs of positive (LR+) and negative (LR−) test results and estimates of 95% CI are shown in Tables 9 and 10. The values for LR+ (ratio of the true-positive rate divided by the false-positive rate) were high (>10) for both culture and qPCR #1. The LR+ for culture indicated that a positive test result was about 75 times more likely to come from an *R. salmoninarum*-infected fish than from an uninfected fish, and the LR+ for qPCR #1 indicated that a positive test result was about 50 times more likely to occur in an infected than in an uninfected fish (Table 9). Lower LR+ values (≤ 5) were obtained for nPCR and smear DFAT (Table 9). The LR+ for ELISA ranged from 2.5 to 188, with LR+ values more than 10 at OD cut-off values ≥ 0.068 (Table 10). The LR− value (ratio of the false-negative rate divided by the true-negative rate) for ELISA was ≤ 0.3 at all cut-

Table 7 Within-run and between-run repeatability of positive assay results for detection of *Renibacterium salmoninarum* isolate GL-64 seeded into ovarian fluid at three concentrations

Repeatability test	*R. salmoninarum* assay					
	Mean % positive results ± SD (% CV[a])					
R. salmoninarum culture concentration $\log_{10}$ CFU mL^{-1}	MF-FAT[b]	ELISA[c]	nPCR[b]	qPCR #1[c]	qPCR #2[c]	qPCR #3[c]
Within-run						
4.0	100 (0)	51 ± 39 (76)	96 ± 6 (6)	82 ± 6 (7)	99 ± 1 (1)	89 ± 6 (7)
5.0	ND	95 ± 8 (8)	ND	100 ± 1 (1)	98 ± 2 (2)	99 ± 2 (2)
6.7	ND	100 (0)	ND	100 (0)	100 (0)	100 (0)
Between-run						
4.1	100 (0)	14 ± 9 (64)	98 ± 5 (5)	88 ± 3 (3)	99 ± 2 (2)	97 ± 4 (4)
5.2	ND	80 ± 6 (8)	ND	99 ± 1 (1)	100 (0)	99 ± 1 (1)
6.4	ND	100 (0)	ND	100 (0)	99 ± 2 (2)	99 ± 2 (2)

ND, not done.
[a]Per cent coefficient of variation (% CV) = (SD/mean) × 100.
[b]Within-run: 10 replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated five times. Between-run: five replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated 10 times (10 separate runs).
[c]Within-run: Thirty replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated 10 times. Between-run: 10 replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated 30 times (30 separate runs).

Table 8 Within-run and between-run repeatability of positive assay results for detection of *Renibacterium salmoninarum* isolate GL-64 seeded into homogenized kidney tissue at three concentrations. Data for qPCR #3 are not shown; this assay did not detect Rs in any samples during within-run testing and only in a single sample at the highest *R. salmoninarum* concentration during between-run testing

Repeatability test	*R. salmoninarum* assay				
	Mean% positive results ± SD (% CV[a])				
R. salmoninarum culture concentration $\log_{10}$ CFU mL^{-1}	Smear FAT[b]	ELISA[c]	nPCR[b]	qPCR #1[c]	qPCR #2[c]
Within-run					
3.4	10 ± 10 (100)	86 ± 24 (28)	88 ± 5 (6)	41 ± 10 (24)	67 ± 7 (10)
5.1	90 ± 7 (8)	71 ± 47 (66)	ND	99 ± 2 (2)	98 ± 3 (3)
6.3	100[d] (0)	100 (0)	ND	100 ± 1 (1)	99 ± 2 (2)
Between-run					
3.7	16 ± 18 (113)	89 ± 9 (10)	100 (0)	40 ± 7 (18)	76 ± 7 (9)
5.8	98 ± 5 (5)	85 ± 5 (6)	ND	100 (0)	99 ± 1 (1)
6.5	ND	100 (0)	ND	100 (0)	99 ± 2 (2)

ND, not done.
[a]Per cent coefficient of variation (% CV) = (SD/mean) × 100.
[b]Within-run: 10 replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated five times. Between-run: five replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated 10 times (10 separate runs).
[c]Within-run: thirty replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated 10 times. Between-run: 10 replicate samples of *R. salmoninarum* seeded into the matrix at each concentration were tested at a given time; testing was repeated 30 times (30 separate runs).
[d]10 replicate samples at this concentration tested once rather than five times by smear FAT.

off values, indicating that an ELISA OD value less than 0.100 was about one-third (or less) as likely to occur in an infected vs. an uninfected fish (Table 10). Low LR− values (<0.1) were calculated at ELISA cut-off OD values ≤ 0.068. The LR− values for smear DFAT, culture, qPCR #1 and nPCR were ≥ 0.3 (Table 9). The DOR (LR+/LR−) was calculated for each assay (Tables 9 & 10) as a measure of the chance of a tested fish being misclassified as positive or negative rather than being correctly classified (negative discrimination). The DOR values for all assays were >1, indicating greater odds of tested fish being correctly classified than misclassified. The

Table 9 Estimated diagnostic sensitivity and specificity and likelihood ratios for positive and negative results of four assays for detection of *Renibacterium salmoninarum* in homogenized kidney tissue from juvenile Chinook salmon that had been injected intraperitoneally with 1.1×10^6 *R. salmoninarum* per fish 15 days before sampling (149 fish) or left untreated (100 fish). The *R. salmoninarum*-injected fish were considered true positives, and the uninjected fish were considered true negatives

Assay	Number of fish				Diagnostic sensitivity[a] (95% CI)	Diagnostic specificity[b] (95% CI)	Likelihood ratio positive result[c] (95% CI)	Likelihood ratio negative result[d] (95% CI)	Diagnostic odds ratio[e]
	TP	FN	TN	FP					
Culture	56	93	100	0[f]	0.38 (0.30, 0.46)	1.00 (0.95, 1.00)	76.1 (4.8, 1217)	0.63 (0.56, 0.71)	121
Smear FAT	113	36	85	15	0.76 (0.68, 0.82)	0.85 (0.77, 0.91)	5.1 (3.1, 8.1)	0.28 (0.21, 0.38)	18
nPCR	30	119	90	10	0.20 (0.14, 0.27)	0.90 (0.83, 0.94)	2.0 (1.0, 3.9)	0.89 (0.80, 0.98)	2
qPCR #1	37	112	100	0	0.25 (0.19, 0.32)	1.00 (0.95, 1.00)	50.5 (3.1, 813)	0.75 (0.69, 0.83)	67

TP, true positives; FN, false negatives; TN, true negatives; FP, false positives.
[a]Diagnostic sensitivity (true positive rate) = (true positives)/(true positives + false negatives).
[b]Diagnostic specificity (true negative rate) = (true negatives)/(false positives + true negatives).
[c]Likelihood ratio of positive result = sensitivity/(1 − specificity).
[d]Likelihood ratio of negative result = (1 − sensitivity)/specificity.
[e]Diagnostic odds ratio = likelihood ratio of positive result/likelihood ratio of negative result.
[f]In case of zero values, all cells were corrected for continuity by adding 0.5.

highest DOR values (≥ 250), indicative of highest accuracy of positive and negative results for the tested population, were obtained for ELISA (Table 10), with the maximum DOR value (2686) observed at the cut-off OD value of 0.072. The lowest DOR value (2) was observed for nPCR (Table 9).

The results from the quantitative and semi-quantitative tests indicated that the majority of *R. salmoninarum*-injected fish testing positive had low to moderate infection levels (Table 11). Among fish testing positive by culture, 71% had *R. salmoninarum* levels $<1 \times 10^4$ CFU g^{-1} and 59% of fish testing positive by qPCR #1 showed *R. salmoninarum* levels $<1 \times 10^4$ cells g^{-1}. By smear DFAT, samples from 96% of positive fish had less than 10 *R. salmoninarum* cells detected in the 100 microscope fields examined per smear. By ELISA testing, 97% or 98% of positive fish showed low to moderate *R. salmoninarum* antigen levels (OD ≤ 0.199). The highest values obtained from *R. salmoninarum*-injected fish by each of these assays were the following: culture (9.9×10^4 CFU g^{-1}), qPCR #1 (2.1×10^6 cells g^{-1}), smear DFAT (12 cells 100 per fields), and ELISA (OD 1.199). Fish in the uninjected group that tested positive by smear DFAT showed ≤ 3 cells 100 per fields, and ELISA OD values for all fish in the uninjected group were ≤ 0.070.

Concordance analysis of positive and negative results from testing of kidney tissues from the *R. salmoninarum*-injected and uninjected fish is shown in Table 12. The strength of agreement between assays beyond chance, as expressed by the κ statistic, ranged from slight to moderate. Moderate agreement of results ($\kappa = 0.41$–0.60) was observed between culture and qPCR #1, culture and ELISA (at ELISA OD cut-off values of 0.095 and 1.00), and smear DFAT and ELISA (at ELISA OD cut-off values ≥ 0.072). With the exception of smear DFAT (which showed the highest concordance of results with ELISA at the 0.072 cut-off OD), concordance of positive and negative results between ELISA and other assays generally increased as ELISA cut-off OD values were raised.

Correlation analysis of *R. salmoninarum* intensity values obtained by the quantitative and semi-quantitative assays from kidney tissue testing of the *R. salmoninarum*-injected fish showed significant correlation ($r_s \geq 0.51$, $P \leq 0.001$) of values between culture and ELISA, culture and qPCR #1, and qPCR #1 and ELISA (Table 13). No significant correlation of values was observed between smear DFAT and any of the other assays ($r_s \leq 0.04$, $P \geq 0.44$) (Table 13).

Discussion

The analyses of specificity, sensitivity and repeatability conducted for our research provided a measure of relative data quality expected from each assay within the limits of the bench-top validation test conditions, and a gauge of each assay's fitness for purpose, which may include diagnosis of clinical disease, surveillance, or inspection (establishing freedom from infection) (AFS-FHS 2010). Inspections and surveillance efforts often rely on use of a screening assay for initial detection (presumptive

Table 10 Estimated diagnostic sensitivity and specificity and likelihood ratios for positive and negative results of ELISA at various negative-positive cut-off values for detection of *Renibacterium salmoninarum* in homogenized kidney tissue from juvenile Chinook salmon that had been injected intraperitoneally with 1.1×10^6 *R. salmoninarum* per fish 15 days before sampling (149 fish) or left untreated (100 fish). The *R. salmoninarum*-injected fish were considered true positives, and the uninjected fish were considered true negatives

Lowest positive ELISA OD	Cut-off calculation[a]/reference	Number of fish				Diagnostic sensitivity[b] (95% CI)	Diagnostic specificity[c] (95% CI)	Likelihood ratio positive result[d] (95% CI)	Likelihood ratio negative result[e] (95% CI)	Diagnostic odds ratio[f]
		TP	FN	TN	FP					
0.064	2 SD[h]	148	1	60	40	0.99 (0.96, 1.00)	0.60 (0.50, 0.69)	2.5 (2.0, 3.2)	0.01 (0.002, 0.08)	250
0.066	3 SD	147	2	85	15	0.99 (0.95, 1.00)	0.85 (0.77, 0.91)	6.6 (4.1, 10.5)	0.02 (0.004, 0.06)	330
0.068	4 SD	145	4	93	7	0.97 (0.93, 0.99)	0.93 (0.86, 0.97)	13.9 (6.8, 28.4)	0.03 (0.01, 0.08)	463
0.070	5 SD	140	9	97	3	0.94 (0.89, 0.97)	0.97 (0.92, 0.99)	31.3 (10.3, 95.6)	0.06 (0.03, 0.12)	522
0.072	6 SD	139	10	100	0[g]	0.93 (0.88, 0.96)	1.00 (0.95, 1.00)	188 (11.8, 2984)	0.07 (0.04, 0.13)	2686
0.074	7 SD	136	13	100	0	0.91 (0.85, 0.95)	1.00 (0.95, 1.00)	184 (11.6, 2920)	0.09 (0.05, 0.15)	2044
0.095	–[h]	111	38	100	0	0.74 (0.67, 0.81)	1.00 (0.95, 1.00)	150 (9.4, 2388)	0.26 (0.20, 0.34)	577
0.100	–[h]	105	44	100	0	0.70 (0.63, 0.77)	1.00 (0.95, 1.00)	142 (8.9, 2261)	0.30 (0.23, 0.38)	473

TP, true positives; FN, false negatives; TN, true negatives; FP, false positives.

[a]Lowest positive ELISA OD values with SD designations were calculated as the mean ELISA OD value of the negative control tissue samples (0.059) plus the value for the number of standard deviations (SD) shown (1 SD = 0.002). The first positive value was set at 0.001 OD units above the negative-positive cut-off.

[b]Diagnostic sensitivity (true positive rate) = (true positives)/(true positives + false negatives).

[c]Diagnostic specificity (true negative rate) = (true negatives)/(false positives + true negatives).

[d]Likelihood ratio of positive result = sensitivity/(1 − specificity).

[e]Likelihood ratio of negative result = (1 − sensitivity)/specificity.

[f]Diagnostic odds ratio = likelihood ratio of positive result/likelihood ratio of negative result.

[g]In case of zero values, all cells were corrected for continuity by adding 0.5.

[h]The 2SD cut-off was recommended by Pascho *et al.* (1987), 0.095 cut-off was recommended by Meyers *et al.* (1993b), the 0.100 cut-off was used by Munson *et al.* (2010).

Table 11 *Renibacterium salmoninarum* infection or antigen levels determined by quantitative tests (culture and qPCR #1) and semi-quantitative tests (smear FAT and ELISA) in homogenized kidney tissue from juvenile Chinook salmon that had been injected intraperitoneally with 1.1×10^6 *R. salmoninarum* per fish 15 days before sampling

Assay	Number of positive fish of 149 (%)	Infection or antigen level category[a]	Number of fish in category (% of positive fish)
Culture	56 (38)	Log_{10} 2.00–2.99 CFU g^{-1}	23 (41)
		Log_{10} 3.00–3.99 CFU g^{-1}	17 (31)
		Log_{10} 4.00–4.99 CFU g^{-1}	16 (29)
Smear FAT	113 (76)	Log_{10} 0.00–0.99 cells 100 fields^{-1}	109 (96)
		Log_{10} 1.00–1.99 cells 100 fields^{-1}	3 (4)
qPCR #1	37 (25)	Log_{10} 2.00–2.99 cells g^{-1}	6 (16)
		Log_{10} 3.00–3.99 cells g^{-1}	16 (43)
		Log_{10} 4.00–4.99 cells g^{-1}	7 (19)
		Log_{10} 5.00–5.99 cells g^{-1}	5 (14)
		Log_{10} 6.00–6.99 cells g^{-1}	3 (8)
ELISA			
Cut-off OD 0.064[b]	148 (99)	Low (OD 0.064–0.199	82 (55)
		Moderate (OD 0.200–0.999)	63 (43)
		High (OD $\geq$1.000)	3 (2)
Cut-off OD 0.072	139 (93)	Low (OD 0.072–0.199	73 (53)
		Moderate (OD 0.200 – 0.999)	63 (45)
		High (OD $\geq$ 1.000)	3 (2)
Cut-off OD 0.095	111 (74)	Low (OD 0.095–0.199	45 (40)
		Moderate (OD 0.200–0.999)	63 (57)
		High (OD $\geq$1.000)	3 (3)
Cut-off OD 0.100	105 (70)	Low (OD 0.100–0.199	39 (37)
		Moderate (OD 0.200–0.999)	63 (60)
		High (OD $\geq$1.000)	3 (3)

[a]Values for infection or antigen level categories were: culture, colony forming units (CFU) g^{-1}; qPCR #1, *R. salmoninarum* cells g^{-1}; smear FAT, *R. salmoninarum* cells per 100 microscope fields (1000× magnification); ELISA, $OD_{405\ nm}$.
[b]For explanation of ELISA OD cut-off values, see Table 10.

test) followed by a confirmatory test based on a different diagnostic principle. In the absence of fully validated diagnostic methods, a variety of assays have been recommended for screening and confirmation of *R. salmoninarum*, and these have included some of the assays tested in this study. The American Fisheries Society-Fish Health Section *Blue Book* (AFS-FHS 2010) recommends FAT, ELISA, culture (the latter followed by biochemical or immunological testing), or PCR for the confirmation of clinical cases presumptively diagnosed by Gram staining. For detecting subclinical infections, the *Blue Book* suggests polyclonal or monoclonal ELISA for screening and PCR for confirmation of *R. salmoninarum* in kidney tissues and the use of PCR or MF-FAT or both for the detection of the bacterium in ovarian fluid. The *Blue Book* recommendation for inspections is FAT for screening (via smears of kidney tissue or smears of pelleted material from ovarian fluid), and culture or nPCR for confirmation. For anadromous salmonids regularly monitored by ELISA, qPCR, or MF-FAT, however, additional testing by FAT is not required. The Fisheries and Oceans Canada *Manual of Compliance* (Fisheries & Oceans Canada 1984; revised 2004) recommends Gram stained kidney or lesion tissue as presumptive evidence of BKD and immunodiffusion or FAT for confirmation. Prior to delisting of BKD by the World Organization for Animal Health (OIE), the recommended tests in the *Manual of Diagnostic Tests for Aquatic Animals* (OIE 2003) were similar to those in the *Blue Book*.

Tests of analytical specificity conducted for the current research indicated that each of the immunological and molecular assays could detect all 11 *R. salmoninarum* isolates from seven salmonid species and different geographic locations in North America and Europe. This is not surprising, considering the limited serological or genetic variation reported among *R. salmoninarum* isolates from diverse geographic locations and salmonid species (Getchell, Rohovec & Fryer 1985; Wiens & Kaattari 1989; Starliper 1996; Grayson *et al.* 1999). Analytical specificity testing with 17 non-target bacterial species did not reveal significant cross-reactivity with either phylogenetically related organisms or other common fish pathogens, including some bacteria that previously had been reported to cross-react in certain immunological

Table 12 Observed agreement (concordance) of positive and negative results between assays for detection of *Renibacterium salmoninarum* in homogenized kidney tissue from juvenile Chinook salmon that had been injected intraperitoneally with 1.1×10^6 *R. salmoninarum* per fish 15 days before sampling (149 fish) or left untreated (100 fish). The κ statistic is the ratio of the observed agreement beyond chance to the maximum possible agreement beyond chance

Assay comparison	Observed % agreement	κ value	Strength of agreement[a]
Culture and smear FAT	63	0.27	Fair
Culture and nPCR	79	0.33	Fair
Culture and qPCR #1	88	0.59	Moderate
Culture and ELISA (OD 0.064 cut-off)[b]	47	0.17	Slight
Culture and ELISA (OD 0.72 cut-off)	67	0.37	Fair
Culture and ELISA (OD 0.095 cut-off)	75	0.47	Moderate
Culture and ELISA (OD 0.100 cut-off)	78	0.51	Moderate
Smear FAT and nPCR	56	0.13	Slight
Smear FAT and qPCR #1	58	0.17	Slight
Smear FAT and ELISA (OD 0.064 cut-off)	69	0.38	Fair
Smear FAT and ELISA (OD 0.72 cut-off)	78	0.56	Moderate
Smear FAT and ELISA (OD 0.095 cut-off)	74	0.48	Moderate
Smear FAT and ELISA (OD 0.100 cut-off)	71	0.42	Moderate
nPCR and qPCR #1	84	0.37	Fair
nPCR and ELISA (OD 0.064 cut-off)	37	0.06	Slight
nPCR and ELISA (OD 0.72 cut-off)	51	0.10	Slight
nPCR and ELISA (OD 0.095 cut-off)	61	0.17	Slight
nPCR and ELISA (OD 0.100 cut-off)	61	0.17	Slight
qPCR #1 and ELISA (OD 0.064 cut-off)	39	0.11	Slight
qPCR #1 and ELISA (OD 0.72 cut-off)	59	0.24	Fair
qPCR #1 and ELISA (OD 0.095 cut-off)	69	0.35	Fair
qPCR #1 and ELISA (OD 0.100 cut-off)	72	0.38	Fair

[a]Strength of agreement (Smith 2006): κ 0 = no better than chance; κ 0.01–0.20 = slight; κ 0.21–0.40 = fair; κ 0.41–0.60 = moderate; κ 0.61–0.80 = substantial; κ 0.81–0.99 = almost perfect; κ 1.00 = perfect.
[b]For explanation of ELISA OD cut-off values, see Table 10.

Table 13 Correlation between values obtained by culture, qPCR #1, ELISA, and smear FAT by testing of homogenized kidney tissue from juvenile Chinook salmon that had been injected intraperitoneally with 1.1×10^6 *Renibacterium salmoninarum* per fish 15 days before sampling. Values compared were: culture, colony forming units g^{-1}; qPCR #1, *R. salmoninarum* cells g^{-1}; ELISA, $OD_{405\ nm}$; smear FAT, *R. salmoninarum* cells per 100 microscope fields (1000× magnification)

Comparison	r_s[a]	*P*	*n*
Culture and smear FAT	0.04	0.79	46
Culture and qPCR #1	0.61	0.0003	31
Culture and ELISA	0.60	<0.0001	56
Smear FAT and ELISA	−0.02	0.87	113
Smear FAT and qPCR #1	−0.15	0.44	30
qPCR #1 and ELISA	0.51	0.001	37

[a]r_s: Spearman's rank correlation coefficient.

or molecular assays for *R, salmoninarum* (see Pascho *et al.* 2002 for a review). The apparent borderline reactivity of qPCR #2 with five non-target bacterial species was determined to be caused by low-level DNA contamination, which may have been facilitated by this assay's use of primer and probe sequences located on the *msa* gene between the first-round forward and reverse primers utilized for the nPCR assay tested in this study (Powell *et al.* 2005). The borderline positive results obtained during ELISA testing of the highest concentrations of *Pseudomonas fluorescens* and *Vibrio ordalii* suspended in saline would have been eliminated using a slightly less conservative negative–positive cut-off value that reduced the occurrence of false-positive results.

The results of analytical sensitivity testing with 11 different *R. salmoninarum* isolates presented a measure of the effects of different seeding matrices on assay performance. For example, the analytical sensitivity of qPCR in the PBS matrix may have been affected by reduced DNA extraction efficiency. The efficiency is likely to be higher in field samples containing host DNA than in samples seeded into PBS owing to the protective 'carrier' effect of the host DNA (Sambrook, Fritsch & Maniatis 1989). Thus, seeded ovarian fluid and (especially) kidney samples could be expected to yield higher amounts of *R. salmoninarum* DNA than seeded PBS after extraction. The qPCR test results for ovarian fluid, and kidney results for qPCRs #1 and #2, supported this hypothesis.

The low rates ($\leq 33\%$ of isolates at each seeding level) of *R. salmoninarum* detection by qPCR #3 in

the kidney tissue matrix at seeding concentrations as high as $\log_{10}$ 6 bacteria g^{-1} suggested PCR inhibition. Because this PCR has been used successfully by other authors to detect *R. salmoninarum* in kidney tissue (Rhodes *et al.* 2006; Sandell & Jacobson 2011), we sought to determine the cause(s) of the poor performance of this assay in our tests. The protocol used by the laboratory that designed qPCR #3 included several reagents (L.D. Rhodes, Northwest Fisheries Science Center, National Oceanic and Atmospheric Administration, personal communication) that were not described in the published protocol (Rhodes *et al.* 2006). Preliminary evaluation of the protocol differences revealed that substitution of TaqMan® Gene Expression Master Mix for the TaqMan® Universal Master Mix (Applied Biosystems) used in our study enabled qPCR #3 to detect *R. salmoninarum* seeded into kidney tissue homogenates. We also ran a preliminary comparison of the two master mix formulations with qPCR #1 and observed an average 3 C_q shift downward in seeded kidney tissues (data not shown), suggesting an approximate 10-fold increase in sensitivity for qPCR #1 with the Gene Expression Master Mix (Pfaffl 2004). These findings demonstrate the importance of complete description of qPCR protocols in publications; guidelines on minimum information required for publication of qPCR experiments have been presented (Bustin *et al.* 2009).

The findings from the PCR master mix comparisons illustrate the value of continual monitoring and evaluation of the performance of established assays, and empirical testing of technical modifications as needed to improve assay efficiency (OIE 2009). The test results suggested that inclusion of an internal positive control (IPC) in the qPCR procedures would be useful for the detection of inhibition (Purcell *et al.* 2011). An IPC assay targets an exogenous artificial template unrelated to the specific pathogen target, and IPC primers and probes are spiked into each PCR along with the artificial template. However, the addition of an IPC creates a multiplex reaction that can reduce sensitivity of a qPCR assay, so the effect of the IPC on analytical sensitivity should be empirically tested (Purcell *et al.* 2011).

Another factor that likely contributed to the decreased analytical sensitivity of the PCRs in comparison with culture or MF-FAT was the lower sample weights or volumes used by the PCR assays, which decreased the total numbers of bacteria present for detection by these assays. For kidney tissue, the initial tissue weight processed from each sample for culture (500 mg) was about 6–10 times greater than the weight (48.6–86.4 mg) processed from each sample for PCR testing. Furthermore, after processing, the calculated weight of each original kidney sample that was inoculated into each culture plate (10 mg) was about 5–8 times higher than that (1.2–2.1 mg) tested by PCR. For ovarian fluid, the difference was even greater. The volume of ovarian fluid from each seeded sample that was inoculated into each culture plate (100 μL) was two times greater than the volume (50 μL) taken from each sample for DNA extraction for PCR testing, and about 80 times greater than the calculated 1.25 μL sample volume that was actually amplified by PCR. In addition, culture plates were inoculated in triplicate, further increasing the weight or volume of each sample tested. Although qPCR samples were tested in duplicate, agreement (positive result) between both replicate samples was required for the sample to be considered positive for *R. salmoninarum*. For culture, a single verified *R. salmoninarum* colony on one of the three replicate plates was considered a positive result. For nPCR, samples were not tested in replicate unless an equivocal result was obtained. The MF-FAT was not tested in replicate, but the 500-μL volume tested from each seeded PBS or ovarian fluid sample was about 400 times greater than the calculated volume amplified by PCR, and the filtration step included in MF-FAT further concentrated the bacteria. Addition of a concentration step by filtration or centrifugation of fluid samples before DNA extraction could increase sensitivity of the PCR assays, as has been demonstrated for other fish pathogens (Hallett & Bartholomew 2006; Griffin *et al.* 2009, 2011).

The analytical sensitivity of the smear DFAT in our tests was also affected by the relatively small (unquantified) amount of kidney tissue homogenate applied in a thin film to a 100.5-mm^2 area of a slide and subsequently examined. For our procedure, a total area of about 4.7 mm^2 of the smear was examined by epifluorescence microscopy (100 defined fields at 1000× magnification), and the analytical sensitivity was on average $\log_{10}$ 4.6 cells g^{-1}. This is similar to the estimate by Bullock, Griffin & Stuckey (1980) that $\log_{10}$ 4 *R. salmoninarum* cells g^{-1} of kidney tissue are needed for a positive DFAT smear. In comparison, for a more quantitative smear DFAT procedure (qFAT) developed by Cvitanich (2004), the

smear is applied to a 525-mm^2 area of a slide, and an area of up to 25 mm^2 (1000 defined fields at 1000× magnification) is examined for *R. salmoninarum*. Rhodes *et al.* (2006) estimated the limit of detection of the qFAT assay to be between $\log_{10}$ 3.5 and $\log_{10}$ 4.4 cells g^{-1} when 400 defined fields at 1000× magnification were examined per slide (total area not reported). Freezing of the kidney tissue homogenates and the slight dilution necessitated by seeding the homogenates with *R. salmoninarum* suspended in saline may have contributed to partial wash-off of some smears in our DFAT tests. Cvitanich (2004) cautioned against freezing or dilution of tissue prior to making smears to prevent possible smear wash-off and reduction in sensitivity.

The low analytical sensitivity observed for ELISA in the seeded matrices in comparison with culture was not unexpected. Other researchers have estimated the limits of polyclonal ELISAs for the detection of *R. salmoninarum* cells in kidney homogenates to be about $\log_{10}$ 6 cells mL^{-1} (Bandín *et al.* 1996) or $\log_{10}$ 7 cells g^{-1} (Jansson *et al.* 1996) when the homogenates were seeded with cells from washed or diluted cultures with much of the soluble antigen likely removed. Jansson *et al.* (1996) reported higher sensitivity for the detection of *R. salmoninarum* antigens in naturally infected fish, with kidney tissues estimated to contain $\log_{10}$ 3 *R. salmoninarum* cells g^{-1} testing positive by polyclonal ELISA.

Renibacterium salmoninarum quantity estimates obtained by culture, MF-FAT and the three qPCRs in the seeded matrices revealed additional information about factors affecting analytical sensitivity and quantitative abilities of these assays. The larger amount (weight or volume) of each sample tested by culture and MF-FAT enabled greater accuracy of *R. salmoninarum* quantity estimates at lower seeding concentrations than was possible for the qPCRs. The amplification patterns in matrices seeded with serially diluted bacteria indicated that efficiency of all three qPCRs was highest in seeded ovarian fluid, and suggested a need for more optimization of the assays for detection and quantification of bacteria in kidney tissue samples. In practice, qPCR amplification efficiency is rarely 100%, resulting in small statistical errors that affect the accuracy of quantification (Bustin 2004). Although the statistical errors have minimal effect when the starting copy number is high, these errors affect both the accuracy and precision of quantification when the starting copy number is less than $\log_{10}$ 3, near the limit of detection (Bustin 2004). Our data indicated that stochastic amplification of very low *R. salmoninarum* DNA copy numbers resulted in over-estimation of *R. salmoninarum* counts by the qPCRs at the lower seeding levels ($\leq \log_{10}$ 3, depending on the matrix).

Within the operating range of the qPCRs, there was less than a 1 $\log_{10}$ difference at a given seeding level in quantity estimates between the qPCRs that detect sequences of the *msa* gene (qPCR #1 and #2) and the qPCR that detects a sequence of the single-copy abc transporter permease gene (PCR #3), despite the use of *R. salmoninarum* isolates with differing *msa* gene copy numbers in the tests. In contrast to the conclusion of Sandell & Jacobson (2011), our results suggested that in practical application any of the three qPCRs could be used for estimating *R. salmoninarum* quantities and that factors such as extraction and PCR efficiencies, pipetting errors, or starting *R. salmoninarum* DNA copy numbers may have more effect on quantification accuracy.

Similar to the findings of Elliott & Barila (1987), our results showed high correlation between *R. salmoninarum* counts obtained by culture and MF-FAT, with MF-FAT counts higher (but within 1 $\log_{10}$ of culture counts) at each seeding level. These data are indicative of detection of intact but non-viable or non-culturable bacteria by MF-FAT (Elliott & Barila 1987).

As expected, the tests of within-run and between-run repeatability for each assay showed increasing rates and consistency of *R. salmoninarum* detection as seeding concentrations increased above the limits of detection determined by analytical sensitivity testing in each matrix. These tests illustrate the difficulty associated with interpretation of test results when *R. salmoninarum* concentrations are at or below threshold levels for consistent detection. When the concentration of a target analyte in a sample is low (e.g. DNA copy number, bacterial cell number or soluble antigen molecule concentration), its occurrence may follow a random (Poisson) distribution, and the analyte may be detected in some replicates but not others.

Tests of diagnostic sensitivity and specificity with samples obtained from experimentally infected animals demonstrated the influence that additional variables associated with such testing

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can have on assay performance. Whereas culture demonstrated the highest analytical sensitivity for *R. salmoninarum* detection in the pasteurized seeded kidney tissue matrix, ELISA and smear DFAT showed the highest estimated diagnostic sensitivity in kidney tissue from *R. salmoninarum*-injected fish. Cultures were inoculated into triplicate SKDM plates at a given dilution for experiments with seeded samples, but only a single plate was inoculated at each dilution for the tissue samples taken from experimentally infected fish. In the challenge experiment, the ability to detect *R. salmoninarum* in cultures may have been obscured or its growth inhibited by the presence of contaminating organisms that were observed on some plates inoculated with kidney tissue homogenates, despite the use of SKDM medium with antibacterial and antifungal agents added. Although the utilization of SKDM reduces the growth of contaminating organisms, it is not truly selective and does not completely eliminate the occurrence of contamination (Austin & Rayment 1985; Sakai *et al.* 1987; Gudmundsdóttir, Helgason & Benediktsdóttir 1991; Olsen *et al.* 1992; Sakai & Kobayashi 1992; Balfry, Albright & Evelyn 1996). Additionally, failure of SKDM to support the growth of *R. salmoninarum* that was culturable on KDM2 has been reported in some instances (Olsen *et al.* 1992; Hirvelä-Koski *et al.* 2006).

The relatively high diagnostic sensitivity of ELISA in our study may reflect not only the accumulation of soluble antigen produced by metabolizing bacteria in fish (Turaga, Wiens & Kaattari 1987), but also the capacity of ELISA to detect soluble antigen that circulates into kidney tissues from infection sites in non-sampled tissues (Pascho *et al.* 1987; Elliott & Pascho 2001). This possibility is supported by the work of Metzger *et al.* (2010), in which BKD lesions were identified by histopathology in the pancreatic interstitium, spleen capsule and liver of some juvenile Chinook salmon 14 days after intraperitoneal injection with *R. salmoninarum*; the injected fish were from the same Wisconsin stock as those used in the current study. The ability of both ELISA and FAT to detect non-viable *R. salmoninarum* may have also contributed to higher diagnostic sensitivity in comparison with culture. Pascho *et al.* (1997) demonstrated persistence of high levels of *R. salmoninarum* antigen for more than 110 days after intraperitoneal injection of rainbow trout, *Oncorhynchus mykiss* (Walbaum), with killed *R. salmoninarum* cells. Studies of salmonid populations suspected of harbouring subclinical *R. salmoninarum* infections have suggested that presumptive diagnoses of *R. salmoninarum* from kidney samples by DFAT in fish testing negative by culture may represent the presence of low numbers of non-viable or non-culturable bacteria in some cases (Evelyn *et al.* 1981; Teska *et al.* 1995; Cvitanich 2004). The lower diagnostic sensitivity of nPCR and qPCR #1 (the only qPCR tested) in comparison with culture was likely related to the smaller sample weights tested by PCR and possible issues with PCR efficiency, as previously discussed.

Tissues from experimentally infected fish produced the highest estimates of diagnostic specificity (100%) for culture, qPCR #1, and for ELISA at negative–positive cut-off values $\geq$OD 0.72. For nPCR, it is uncertain whether the bands indicative of a 320-bp PCR product that were detected in 10% of control fish represented spurious bands or target DNA, because we did not attempt sequencing of the PCR products. Several factors may have contributed to the detection of low numbers of presumptive *R. salmoninarum* cells by DFAT in 15% of the uninjected fish. Cross-reactivity of non-*R. salmoninarum* bacterial species has been reported with some antisera used for FATs to detect the bacterium (Bullock *et al.* 1980; Evelyn *et al.* 1981; Austin *et al.* 1985; Yoshimizu, Ji & Kimura 1987; Foott *et al.* 1992; Brown *et al.* 1995; Teska *et al.* 1995), and it is possible that cross-reacting bacteria were present in kidney tissues of some experimental fish, although DFAT testing of contaminating organisms on SKDM culture plates inoculated with kidney samples did not reveal any bacteria that cross-reacted with the antiserum used. Other possible reasons for false-positive FAT results include misinterpretation of some fluorescing particles as *R. salmoninarum* by the observer or cross-contamination of FAT smears during rinse steps (Evelyn *et al.* 1981; Meyers *et al.* 1993a). Testing of samples by DFAT and other assays was performed by a blinded design, and DFAT slides from *R. salmoninarum*-challenged and control fish were placed together in slide carriers and dipped into containers during rinse steps. We have since modified the rinse steps such that slides are placed on racks with space between slides and rinsed carefully with a squirt bottle to reduce the potential

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for cross-contamination. Finally, the possibility that the nPCR and DFAT positives represented the detection of *R. salmoninarum* present in some control fish at very low levels cannot be completely excluded.

The effect on diagnostic sensitivity and specificity of changing ELISA negative–positive cut-off values illustrates the importance of careful selection of threshold values for a quantitative or semi-quantitative assay to fit the purpose for which the assay is intended. For example, if an assay is to be used to demonstrate freedom from infection in a population or for confirmation of a clinical diagnosis, it may be more important to have high diagnostic specificity to minimize false positives, but if the purpose of the assay is disease eradication or elimination of infection from a population, high diagnostic sensitivity may be more essential for minimization of false negatives (Greiner, Pfeiffer & Smith 2000; Caraguel *et al.* 2011). In populations where an assay is used for surveillance to estimate infection prevalence, decreasing the overall misclassification of false positives and false negatives may be the priority, regardless of the infection and/or disease status (Caraguel *et al.* 2011). Among the measures of diagnostic accuracy, the DOR may be one of the most useful measures for the selection of assay cut-off values to minimize misclassification of positive or negative results, independent of disease or infection prevalence (Caraguel *et al.* 2011). For the test population in our study, an ELISA OD cut-off value of 0.072 yielded the highest DOR, and calculation of other indices of assay performance including ROC cut-off point optimization and Youden's J index (Thrusfield 2005) resulted in the same optimum cut-off OD value (data not shown). However, these performance measures assume equal importance of diagnostic sensitivity and specificity, and do not factor in the economic or population cost of misclassification (Caraguel *et al.* 2011). As an example, in the implementation of ELISA-based culling of egg lots from *R. salmoninarum*-infected female salmonids to reduce the risk of vertical transmission of the pathogen in hatcheries, higher or lower ELISA cut-off OD values may be selected based on population size and infection prevalence, to maximize the benefits of this BKD control strategy without jeopardizing hatchery production goals (Meyers *et al.* 1993b; Munson *et al.* 2010). In situations where separate screening and confirmatory assays are used for population testing, the cut-off value for the screening test should be set to ensure high diagnostic sensitivity (>95%), but specificity can be lower than that of the confirmatory test (Elder *et al.* 1997). Thus, a negative result for a screening assay should indicate that the sample has a high probability of being free of the pathogen, whereas a positive test result might reflect only the need for confirmatory testing.

Reports of discrepancies in positive and negative results among different *R. salmoninarum* diagnostic assays are common and can make confirmation of test results difficult (e.g. Evelyn *et al.* 1981; Pascho *et al.* 1987, 1998; Meyers *et al.* 1993a; Teska *et al.* 1995; Bruno *et al.* 2007; Faisal & Eissa 2009; Nance *et al.* 2010; Sandell & Jacobson 2011). A variety of factors have been hypothesized to impact assay concordance. Non-uniform *R. salmoninarum* distribution in infected fish and differences in the analytes detected by the assays can affect agreement of results (Meyers *et al.* 1993a). Bacteria in focal granulomatous lesions will not be detected by FAT or culture if the tissue containing intact bacterial cells (for FAT) or viable cells (for culture) is not included in the sample tested. Non-uniform pathogen distribution combined with the small amount of tissue tested can also hamper PCR detection of *R. salmoninarum* DNA. Concordance can also be influenced by differences in *R. salmoninarum* infection stage in fish at the time of sampling, which can affect the amounts of various analytes present (Cvitanich 2004; Faisal & Eissa 2009; Nance *et al.* 2010). In naturally infected salmonid populations, fish representing various stages of infection would be expected to be present at the same time (Jansson *et al.* 1996, 2008; Elliott *et al.* 1997; Faisal & Eissa 2009; Nance *et al.* 2010; Sandell & Jacobson 2011). Experimental exposure of a group of fish to *R. salmoninarum* on a single date as occurred in our study may result in a reduction or skewing of the spectrum of infection stages manifested in the population. Finally, low concordance can be more evident when infection intensity is low. The culture and qPCR results in our challenge experiment indicated that the *R. salmoninarum* levels in the kidneys of most of the *R. salmoninarum*-injected fish were less than $\log_{10}$ 4 bacteria g^{-1} at the time of sampling and, therefore, were near or below the threshold for consistent detection by most of the assays as determined by analytical sensitivity testing.

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In tests of kidney tissue from laboratory-challenged fish in our study, concordance of positive and negative results between assays was moderate or lower (κ = 0.06–0.59). It should be noted that estimates of concordance by the κ statistic can be biased lower (e.g. $\kappa < 0.40$) even with relatively high agreement (e.g. agreement >0.80) when infection prevalence is either very high or low (Feinstein & Cicchetti 1990). The results of concordance analysis of positive and negative results for nPCR and qPCR #1 fit this pattern with high observed agreement (0.84) but a low κ score (0.37); the infection prevalence detected by the two assays was 15% and 16%, respectively.

The significant positive correlations observed in our research between values obtained for qPCR and ELISA, culture and ELISA, and culture and qPCR in tests of tissues from challenged fish were not unexpected. Although qPCR and ELISA measure different *R. salmoninarum* macromolecules and antigen concentration may not accurately reflect the number of cells present (Hamel & Anderson 2002), significant positive correlation between qPCR quantity estimates and ELISA OD values from kidney sample testing, as occurred in our study with laboratory-challenged fish, also has been reported by other researchers for experimentally or naturally infected fish (Powell *et al.* 2005; Chase *et al.* 2006; Jansson *et al.* 2008; Nance *et al.* 2010; Sandell & Jacobson 2011). Correlation between qPCR and ELISA values is usually stronger at higher infection levels, but exceptions have been noted (Sandell & Jacobson 2011). Significant positive correlation between culture counts and qPCR values for *R. salmoninarum* also has been previously described for kidney samples of fish experimentally infected by injection or immersion challenge (Suzuki & Sakai 2007).

The lack of significant correlation between kidney smear DFAT counts and the results of any of the other quantitative or semi-quantitative assays in our laboratory-challenge study was not surprising. The very low numbers of bacteria observed by smear DFAT (<10 bacteria 100 per fields in 96% of DFAT-positive *R. salmoninarum*-challenged fish) were indicative of stochastic, non-quantitative detection of bacteria in the FAT smears. Other research has also shown inconsistent FAT detection and low bacterial counts in kidney samples from naturally infected fish showing polyclonal ELISA OD values less than 1.000 (Meyers *et al.* 1993a; Elliott *et al.* 1997); only 2% of the *R. salmoninarum*-injected fish in our study showed ELISA OD values ≥ 1.000.

Our research did not reveal a single *R. salmoninarum* assay that would likely exhibit perfect diagnostic sensitivity and specificity for testing of fish at all stages of infection in a population, but did identify advantages and disadvantages of each assay for use as a screening or confirmatory test or both. For example, our results were in agreement with previous research (e.g. Meyers *et al.* 1993b) that polyclonal ELISA can be a useful screening assay for kidney tissue with the negative–positive cut-off OD value adjusted according to the intended purpose, but poor concordance with confirmatory assays such as PCR or culture can be expected in cases of low infection intensity, non-uniform pathogen distribution, or antigen persistence in the absence of detectable viable bacteria or intact DNA. Because *R. salmoninarum* soluble antigen levels may not correspond to bacterial load at all stages of infection (Hamel & Anderson 2002), it is difficult to precisely define a limit of detection for ELISA. We did not evaluate the diagnostic sensitivity of polyclonal ELISA for testing ovarian fluid samples, but other studies have indicated that both polyclonal and monoclonal antibody ELISAs lack sensitivity for *R. salmoninarum* screening of ovarian fluid (Pascho *et al.* 1991, 1998; Griffiths, Liska & Lynch 1996); polyclonal ELISAs may not reliably detect *R. salmoninarum* antigen in ovarian fluid until MF-FAT counts exceed $\log_{10}$ 4–5 bacteria mL^{-1} (Pascho *et al.* 1991, 1998).

A strength of PCR is the ability to amplify DNA from low numbers of target organisms present in tested material, and both conventional and quantitative PCRs have been gaining popularity as screening or confirmatory assays for aquatic pathogens for more than a decade (OIE 2003, 2009; AFS-FHS 2005, 2010; Purcell *et al.* 2011). Conversely, a drawback is that verification of positive PCR results by non-molecular tests in field samples from fish populations with low infection prevalence and intensity can be difficult (e.g. Bruno *et al.* 2007; Chambers, Gardiner & Peeler 2008). Precedent for use of DNA sequencing to confirm PCR results for *R. salmoninarum* and other pathogens has been established (Arsan *et al.* 2007; Chambers *et al.* 2008; OIE 2009; AFS-FHS 2010), but distinguishing between *R. salmoninarum* sequences originating from tissue infections and

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laboratory contamination may be problematic owing to low genetic diversity among strains.

Reduction of contamination by the elimination of post-PCR manipulation of products, quantitative abilities, speed, robustness and reproducibility are the advantages of qPCR over nPCR (Bastien, Procop & Reischl 2008; Purcell *et al.* 2011), whereas higher initial startup and operational costs are disadvantages. The qPCR assays are not necessarily more sensitive than 'conventional' PCR assays such as nPCR (Bastien *et al.* 2008). In contrast to the findings of some researchers (Powell *et al.* 2005) but in agreement with others (Chase *et al.* 2006), our study did not demonstrate significantly higher analytical or diagnostic sensitivity for qPCR compared with nPCR. Nevertheless, the higher diagnostic specificity for qPCR than for nPCR resulted in a higher DOR, indicative of greater odds of tested fish being correctly classified as *R. salmoninarum*-positive or *salmoninarum*-negative by qPCR than by nPCR. Our results suggested that any of the three qPCR assays tested would be suitable for *R. salmoninarum* detection in kidney tissue or ovarian fluid, provided that the procedural modifications discussed for qPCR #1 and qPCR #3 were implemented to improve assay performance for kidney sample testing. In comparison, Sandell & Jacobson (2011) showed a higher detection rate by qPCR #1 (53.2%) than by qPCR #3 (21.8%) for nPCR-positive kidney samples from naturally infected Chinook and coho, *Oncorhynchus kisutch* (Walbaum), salmon. In laboratories that also use the nPCR assay of Chase & Pascho (1998), qPCR #2 may show increased susceptibility to laboratory contamination because the primer sequences of the two assays overlap. In agreement with Bustin (2004), our results suggested that *R. salmoninarum* detection by PCR and quantity estimates by qPCR may not be consistent at concentrations $<\log_{10}$ 3 bacteria g^{-1} or mL^{-1}. Because consistency of pathogen detection by PCR can be affected by factors such as the small amount of material tested and by non-uniform pathogen distribution (Purcell *et al.* 2011), an increase in technical replicates may be useful to enhance PCR sensitivity in critical cases (Jansson *et al.* 2008), but this will add to testing costs.

Our research, similar to that of Bullock *et al.* (1980), indicated applicability of DFAT for screening of fish with $>\log_{10}$ 4 *R. salmoninarum* cells g^{-1} in kidney tissues. Like PCR, DFAT sensitivity is affected by the small amount of sample tested and by non-uniform distribution of intact bacteria (Meyers *et al.* 1993a). Both the sensitivity and quantitative abilities of smear DFAT can be increased using qFAT methodology (Cvitanich 2004; Rhodes *et al.* 2006), but this procedure is labour-intensive. The subjective interpretation required for FAT procedures can lead to misclassification of results, as indicated by the relatively low DOR for smear DFAT, which was lower than the DOR values for all other assays except nPCR for *R. salmoninarum* detection in tissues from experimentally infected fish.

The current findings corroborated the results of other research showing high sensitivity of MF-FAT for *R. salmoninarum* detection in fluid samples (Elliott & Barila 1987; Elliott & McKibben 1997), with the mean detection limit ranging about $\log_{10}$ 1–2 bacteria mL^{-1} in our study. In contrast to our results with seeded samples, Pascho *et al.* (1998) demonstrated a higher *R. salmoninarum* detection rate by nPCR than by MF-FAT in ovarian fluid from naturally infected fish; it is unknown whether some of the nPCR detections in that study represented free *R. salmoninarum* DNA rather than DNA in intact cells (Banada *et al.* 2012). An advantage of MF-FAT is its quantitative ability, with reliable quantification observed over a greater *R. salmoninarum* concentration range in comparison with qPCR in our research. Like smear DFAT, however, MF-FAT is labour-intensive, which limits its utility as a screening assay for large sample sets. A relatively new technology, solid-phase laser scanning cytometry (SPC) automates the process of enumerating fluorescence-stained bacteria concentrated on filters and demonstrates higher sensitivity and repeatability than direct fluorescence microscopy (Lemarchand *et al.* 2001; Lisle *et al.* 2004). Preliminary tests in our laboratory have indicated greater sensitivity of SPC than MF-FAT for *R. salmoninarum* detection in fluid samples (McKibben and Elliott, unpublished), but the high cost of instrumentation may make this methodology practical only for larger diagnostic or research laboratories.

While bacteriological culture, employed as the benchmark test in the current research, showed the highest analytical sensitivity among assays for *R. salmoninarum* detection in each seeded matrix (limit of detection between $\log_{10}$ 1–2 bacteria g^{-1} or mL^{-1}), the pasteurization of ovarian fluid and kidney before seeding and the inoculation of

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triplicate culture plates per dilution in the seeding tests undoubtedly contributed to the high analytical sensitivity. Conversely, the presence of contaminating organisms on some culture plates and inoculation of a single plate per kidney sample from each experimentally challenged fish probably contributed to reduced diagnostic sensitivity of this assay. A culture (KDM2) detection limit of $\log_{10}$ 2.5 CFU mL^{-1} was previously estimated for *R. salmoninarum* seeded into non-pasteurized kidney tissue (Paclibare, Evelyn & Albright 1988), and a culture (SKDM) detection limit of $\log_{10}$ 2.7 CFU g^{-1} was estimated for kidney tissue from chum salmon, *Oncorhynchus keta* (Walbaum), fry infected with *R. salmoninarum* (Suzuki & Sakai 2007). Non-uniform distribution of viable bacteria and apparent inhibition of *R. salmoninarum* growth by unknown factors in kidney tissue can also affect culture sensitivity (Evelyn *et al.* 1981; Daly & Stevenson 1988), but the use of homogenized and washed kidney tissue for inoculation of all culture plates, and the incorporation of filter-sterilized *R. salmoninarum*-conditioned medium (Evelyn *et al.* 1990; Teska 1993) to SKDM should have reduced these issues in our study.

Among the assays evaluated in our research, only culture can confirm the viability of *R. salmoninarum*, but the long incubation period (up to 12–19 weeks) required for growth of visible colonies (Benediktsdóttir, Helgason & Gudmundsdóttir 1991) makes culture impractical for use in situations where rapid diagnosis is needed. Reverse transcription (RT) nPCR assays (Cook & Lynch 1999) and, more recently, RT-qPCR assays (Powell *et al.* 2005; Suzuki & Sakai 2007; Halaihel *et al.* 2009; Metzger *et al.* 2010) have been developed to detect the expression of *R. salmoninarum* messenger RNA (mRNA), which has a half-life usually measured in minutes (e.g., von Gabain *et al.* 1983; Belasco *et al.* 1986; Rauhut & Klug 1999) and is considered an indicator of the presence of viable bacteria (Sheridan *et al.* 1998). However, Metzger *et al.* (2010) cautioned that mRNA expression could be affected by differential regulation of *R. salmoninarum* genes during different stages of infection. Suzuki & Sakai (2007) reported good correlation between *R. salmoninarum msa* gene mRNA concentration and culturable cell concentration in kidneys of chum salmon fry challenged by injection; however, the limit for mRNA detection in kidney tissue was about $\log_{10}$ 5.5 copies g^{-1} compared with $\log_{10}$ 4.7 copies g^{-1} for the DNA assay.

To our knowledge, this study represents the most comprehensive effort to date towards completing the bench-top validation of several commonly used *R. salmoninarum* diagnostic assays. For our research, quantification of bacteria in samples by culture provided a standardized measure of viable *R. salmoninarum* for the evaluation of performance characteristics of the non-culture assays. In addition, for preliminary estimates of diagnostic sensitivity and specificity, the use of a reference population of fish hatched and reared in the laboratory from eggs obtained from SPF parents greatly reduced the possibility of results confounded by pre-existing *R. salmoninarum* infections. In the future, it will be useful to obtain more accurate estimates of diagnostic sensitivity and specificity. We have been performing waterborne *R. salmoninarum* challenges of SPF fish to evaluate *R. salmoninarum* detection by various assays over time and at different stages of infection, following a more natural route of infection than injection. Nevertheless, the ideal reference populations for establishing diagnostic sensitivity and specificity consist of naturally infected and uninfected fish (OIE 2009). Because the true infection status of fish in such populations is often unknown, evaluation of diagnostic sensitivity and specificity of an assay in the absence of a gold standard (perfect reference test) may require procedures such as comparison with a reference test of known (but imperfect) diagnostic sensitivity and/or specificity, the use of a composite reference standard or application of latent class modelling (Purcell *et al.* 2011). Finally, for complete validation of a diagnostic assay, reproducibility and ruggedness of the method must be evaluated by testing in multiple laboratories (OIE 2009). We are currently conducting inter-laboratory testing of selected non-culture *R. salmoninarum* assays to obtain measures of reproducibility and ruggedness.

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References

AFS-FHS (2005) *AFS-FHS (American Fisheries Society-Fish Health Section) FHS Blue Book. Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*, 2005 edn. AFS-FHS, Bethesda, MD.

AFS-FHS (2010) *AFS-FHS (American Fisheries Society-Fish Health Section) FHS Blue Book: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*, 2010 edn. AFS-FHS, Bethesda, MD.

Akobeng A.K. (2006) Understanding diagnostic tests 2: likelihood ratios, pre- and post-test probabilities and their use in clinical practice. *Acta Pediatrica* **96**, 487–491.

Alcorn S., Murray A.L., Pascho R.J. & Varney J. (2005) A cohabitation challenge to compare the efficacies of vaccines for bacterial kidney disease (BKD) in chinook salmon *Oncorhynchus tshawytscha*. *Diseases of Aquatic Organisms* **63**, 151–160.

Arsan E.L., Atkinson S.D., Hallett S.L., Meyers T.R. & Bartholomew J.L. (2007) Expanded geographical distribution of *Myxobolus cerebralis*: first detection from Alaska. *Journal of Fish Diseases* **30**, 483–491.

Austin B. & Austin D.A. (1987) *Bacterial Fish Pathogens: Disease in Farmed and Wild Fish*. Ellis Horwood, Chichester, UK.

Austin B. & Rayment J.N. (1985) Epizootiology of *Renibacterium salmoninarum*, the causal agent of bacterial kidney disease of salmonid fish. *Journal of Fish Diseases* **8**, 505–509.

Austin B., Embley T.M. & Goodfellow M. (1983) Selective isolation of *Renibacterium salmoninarum*. *FEMS Microbiology Letters* **17**, 111–114.

Austin B., Bucke D., Feist S. & Rayment J. (1985) A false positive reaction in the indirect fluorescent antibody test for *Renibacterium salmoninarum* with a "coryneform" organism. *Bulletin of the European Association of Fish Pathologists* **5**, 8–9.

Balfry S.K., Albright L.J. & Evelyn T.P.T. (1996) Horizontal transfer of *Renibacterium salmoninarum* among farmed salmonids via the fecal-oral route. *Diseases of Aquatic Organisms* **25**, 63–69.

Banada P.P., Chakravorty S., Shah D., Burday M., Mazzella F.M. & Alland D. (2012) Highly sensitive detection of *Staphylococcus aureus* directly from patient blood. *PLoS ONE* **7**, e31126.

Bandín I., Ellis A.E., Barja J.L. & Secombes C.J. (1993) Interaction between rainbow trout macrophages and *Renibacterium salmoninarum in vitro*. *Fish and Shellfish Immunology* **3**, 25–33.

Bandín I., Heinen P., Brown L.L. & Toranzo A.E. (1996) Comparison of different ELISA kits for detecting *Renibacterium salmoninarum*. *Bulletin of the European Association of Fish Pathologists* **16**, 19–22.

Bastien P., Procop G.W. & Reischl U. (2008) Quantitative real-time PCR is not more sensitive than "conventional" PCR. *Journal of Clinical Microbiology* **46**, 1897–1900.

Belasco J.G., Nilsson G., von Gabain A. & Cohen S.N. (1986) The stability of *E. coli* gene transcripts is dependent on determinants localized to specific mRNA segments. *Cell* **46**, 245–251.

Bell G.R., Higgs D.A. & Traxler G.S. (1984) The effect of dietary ascorbate, zinc, and manganese on the development of experimentally induced bacterial kidney disease in sockeye salmon (*Oncorhynchus nerka*). *Aquaculture* **36**, 293–311.

Benediktsdóttir E., Helgason S. & Gudmundsdóttir S. (1991) Incubation time for the cultivation of *Renibacterium salmoninarum* from Atlantic salmon, *Salmo salar* L., broodfish. *Journal of Fish Diseases* **14**, 97–102.

Brown L.L., Evelyn T.P.T., Iwama G.K., Nelson W.S. & Levine R.P. (1995) Bacterial species other than *Renibacterium salmoninarum* cross-react with antisera against *R. salmoninarum* but are negative for the p57 gene of *R. salmoninarum* as detected by the polymerase chain reaction (PCR). *Diseases of Aquatic Organisms* **21**, 227–231.

Bruno D.W. (1986a) Histopathology of bacterial kidney disease in laboratory infected rainbow trout, *Salmo gairdneri* Richardson, and Atlantic salmon, *Salmo salar* L., with reference to naturally infected fish. *Journal of Fish Diseases* **9**, 523–537.

Bruno D.W. (1986b) Scottish experience with bacterial kidney disease in farmed salmonids between 1976 and 1985. *Aquaculture and Fisheries Management* **17**, 185–190.

Bruno D.W. (1988) The relationship between auto-agglutination, cell surface hydrophobicity and virulence of the fish pathogen *Renibacterium salmoninarum*. *FEMS Microbiology Letters* **51**, 135–139.

Bruno D., Collet B., Turnbull A., Kilburn R., Walker A., Pendrey D., McIntosh A., Urquhart K. & Taylor G. (2007) Evaluation and development of diagnostic methods for *Renibacterium salmoninarum* causing bacterial kidney disease (BKD) in the UK. *Aquaculture* **269**, 114–122.

Bullock G.L., Stuckey H.M. & Mulcahy D. (1978) Corynebacterial kidney disease: egg transmission following iodophore disinfection. *Fish Health News* **7**, 51–52.

Bullock G.L., Griffin B.R. & Stuckey H.M. (1980) Detection of *Corynebacterium salmoninus* by direct fluorescent antibody test. *Canadian Journal of Fisheries and Aquatic Sciences* **37**, 719–721.

Bustin S.A. (2004) Quantification of nucleic acids by PCR. In: *A–Z of Quantitative PCR* (ed. by S.A. Bustin), pp. 3–46. International University Line, La Jolla, CA.

Bustin S.A., Benes V., Garson J.A., Hellmans J., Huggett J., Kubista M., Mueller R., Nolan T., Pfaffl M.W., Shipley G.L., Vandesompele J. & Wittwer C.T. (2009) The MIQE guidelines: minimum information for publication of real-time PCR experiments. *Clinical Chemistry* **55**, 611–622.

Caraguel C.G.B., Stryhn H., Gagné N., Dohoo I.R. & Hammell K.L. (2011) Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: analytical and epidemiological approaches. *Journal of Veterinary Diagnostic Investigation* **23**, 2–15.

Chambers E., Gardiner R. & Peeler E.J. (2008) An investigation into the prevalence of *Renibacterium salmoninarum* in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), and wild fish populations in selected river catchments in England and Wales between 1998 and 2000. *Journal of Fish Diseases* **31**, 89–96.

Chase D.M. & Pascho R.J. (1998) Development of a nested polymerase chain reaction for amplification of a sequence of the p57 gene of *Renibacterium salmoninarum* that provides a highly sensitive method for detection of the bacterium in salmonid kidney. *Diseases of Aquatic Organisms* **34**, 223–229.

Chase D.M., Elliott D.G. & Pascho R.J. (2006) Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. *Journal of Veterinalry Diagnostic Investigation* **18**, 375–380.

Clarridge J.E. III (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews* **17**, 840–862.

Coady A.M., Murray A.L., Elliott D.G. & Rhodes L.D. (2006) Both msa genes in *Renibacterium salmoninarum* are needed for full virulence in bacterial kidney disease. *Applied and Environmental Microbiology* **72**, 2672–2678.

Cook M. & Lynch W.H. (1999) A sensitive nested reverse transcriptase PCR assay to detect viable cells of the fish pathogen *Renibacterium salmoninarum* in Atlantic salmon (*Salmo salar* L.). *Applied and Environmental Microbiology* **65**, 3042–3047.

Cvitanich J.D. (2004) *Renibacterium salmoninarum* bar forms: characterization, occurrence, and evidence of a host response to a *R. salmoninarum* infection. *Journal of Fish Diseases* **27**, 193–211.

Daly J.G. & Stevenson R.M.W. (1988) Inhibitory effects of salmonid tissue on the growth of *Renibacterium salmoninarum*. *Diseases of Aquatic Organisms* **4**, 169–171.

Elder B.L., Hansen S.A., Kellogg J.A., Marsik F.J. & Zabransky R.J. (1997). *Verification and Validation of Procedures in the Clinical Microbiology Laboratory*, Cumitech 31 (coordinating ed. B.W. McCurdy), pp. 18, American Society for Microbiology, Washington, DC.

Elliott D.G. & Barila T.Y. (1987) Membrane filtration-fluorescent antibody staining procedure for detecting and quantifying *Renibacterium salmoninarum* in coelomic fluid of chinook salmon (*Oncorhynchus tshawytscha*). *Canadian Journal of Fisheries and Aquatic Sciences* **44**, 206–210.

Elliott D.G. & McKibben C.L. (1997) Comparison of two fluorescent antibody techniques (FATs) for detection and quantification of *Renibacterium salmoninarum* in coelomic fluid of spawning chinook salmon *Oncorhynchus tshawytscha*. *Diseases of Aquatic Organisms* **30**, 37–43.

Elliott D.G. & Pascho R.J. (2001) Evidence that coded-wire-tagging procedures can enhance transmission of *Renibacterium salmoninarum* in chinook salmon. *Journal of Aquatic Animal Health* **13**, 181–193.

Elliott D.G., Pascho R.J. & Bullock G.L. (1989) Developments in the control of bacterial kidney disease of salmonid fishes. *Diseases of Aquatic Organisms* **6**, 201–215.

Elliott D.G., Pascho R.J., Jackson L.M., Matthews G.M. & Harmon J.R. (1997) *Renibacterium salmoninarum* in spring-summer chinook salmon smolts at dams on the Columbia and Snake Rivers. *Journal of Aquatic Animal Health* **9**, 114–126.

Evelyn T.P.T. (1977) An improved growth medium for the kidney disease bacterium and some notes on using the medium. *Bulletin de l'Office International des Epizooties* **87**, 511–513.

Evelyn T.P.T. (1993) Bacterial kidney disease—BKD. In: *Bacterial Diseases of Fish* (ed. by V. Inglis, R.J. Roberts & N.R. Bromage), pp. 177–195. Halsted Press, New York.

Evelyn T.P.T., Ketcheson J.E. & Prosperi-Porta L. (1981) The clinical significance of immunofluorescence-based diagnoses of the bacterial kidney disease carrier. *Fish Pathology* **15**, 293–300.

Evelyn T.P.T., Ketcheson J.E. & Prosperi-Porta L. (1984a) Further evidence for the presence of *Renibacterium salmoninarum* in salmonid eggs and for the failure of povidone-iodine to reduce the intra-ovum infection rate in water-hardened eggs. *Journal of Fish Diseases* **7**, 173–182.

Evelyn T.P.T., Prosperi-Porta L. & Ketcheson J.E. (1984b). The salmonid egg as a vector of the kidney disease bacterium, *Renibacterium salmoninarum*. In: *Fish Diseases*, 4th COPRAQ Session (ed. by ACIUGRUP), pp. 111–117. Editora ATP, Madrid.

Evelyn T.P.T., Prosperi-Porta L. & Ketcheson J.E. (1986) Persistence of the kidney-disease bacterium, *Renibacterium salmoninarum*, in coho salmon, *Oncorhynchus kisutch* (Walbaum), eggs treated during and after water-hardening with povidone-iodine. *Journal of Fish Diseases* **9**, 461–464.

Evelyn T.P.T., Prosperi-Porta L. & Ketcheson J.E. (1990) Two new techniques for obtaining consistent results when growing *Renibacterium salmoninarum* on KDM2 culture medium. *Diseases of Aquatic Organisms* **9**, 209–212.

Evenden A.J., Grayson T.H., Gilpin M.L. & Munn C.B. (1993) *Renibacterium salmoninarum* and bacterial kidney disease - the unfinished jigsaw. *Annual Review of Fish Diseases* **3**, 87–104.

Faisal M. & Eissa A.E. (2009) Diagnostic testing patterns of *Renibacterium salmoninarum* in spawning salmonid stocks in Michigan. *Journal of Wildlife Diseases* **45**, 447–456.

Feinstein A.R. & Cicchetti D.V. (1990) High agreement but low kappa: I. The problems of two paradoxes. *Journal of Clinical Epidemiology* **43**, 543–549.

Ferguson H.W., ed. (2006) *Systemic Pathology of Fish*, 2nd edn. Scotian Press, London, UK.

Published 2013.

Fisheries and Oceans Canada (1984, revised 2004) *Fish Health Protection Regulations: Manual of Compliance*. Fisheries and Marine Service Miscellaneous Special Publications 31 (revised), Ottawa.

Flaño E., López-Fierro P., Razquin B., Kaattari S.L. & Villena A. (1996) Histopathology of the renal and splenic haemopoietic tissues of coho salmon *Oncorhynchus kisutch* experimentally infected with *Renibacterium salmoninarum*. *Diseases of Aquatic Organisms* **24**, 107–115.

Foott J.S., Starliper C.E., Walker R.L. & Junell D. (1992) *Pseudomonas* isolate gives positive direct fluorescent antibody test using *Renibacterium salmoninarum* antiserum. *Fish Health Section/American Fisheries Society Newsletter* **20**, 2–3.

Fryer J.L. & Lannan C.N. (1993) The history and current status of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in Pacific salmon. *Fisheries Research* **17**, 15–33.

Fryer J.L. & Sanders J.E. (1981) Bacterial kidney disease of salmonid fish. *Annual Review of Microbiology* **35**, 273–298.

von Gabain A., Belasco J.G., Schottel J.L., Chang A.C.Y. & Cohen S.N. (1983) Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 653–657.

Getchell R.G., Rohovec J.S. & Fryer J.L. (1985) Comparison of *Renibacterium salmoninarum* isolates by antigenic analysis. *Fish Pathology* **20**, 149–159.

Grayson T.H., Cooper L.F., Atienzar F.A., Knowles M.R. & Gilpin M.L. (1999) Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. *Applied and Environmental Microbiology* **65**, 961–968.

Greiner M., Pfeiffer D. & Smith R.D. (2000) Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. *Preventive Veterinary Medicine* **45**, 23–41.

Griffin M.J., Pote L.M., Camus A.C., Mauel M.J., Greenway T.E. & Wise D.J. (2009) Application of a real-time PCR assay for the detection of *Henneguya ictaluri* in commercial channel catfish ponds. *Diseases of Aquatic Organisms* **86**, 223–233.

Griffin M.J., Mauel M.J., Greenway T.E., Khoo L.H. & Wise D.J. (2011) A real-time polymerase chain reaction assay for quantification of *Edwardsiella ictaluri* in catfish pond water and genetic homogeneity of diagnostic case isolates from Mississippi. *Journal of Aquatic Animal Health* **23**, 178–188.

Griffiths S.G., Liska K. & Lynch W.H. (1996) Comparison of kidney tissue and ovarian fluid from broodstock Atlantic salmon for detection of *Renibacterium salmoninarum*, and use of SKDM broth culture with Western blotting to increase detection in ovarian fluid. *Diseases of Aquatic Organisms* **24**, 3–9.

Gudmundsdóttir S., Helgason S. & Benediktsdóttir E. (1991) Comparison of three different growth media for primary isolation of *Renibacterium salmoninarum* from Atlantic salmon, *Salmo salar* L., broodfish. *Journal of Fish Diseases* **14**, 89–96.

Gudmundsdóttir S., Helgason S., Sigurjónsdóttir H., Matthíasdóttir S., Jónsdóttir H., Laxdal B. & Benediktsdóttir E. (2000) Measures applied to control *Renibacterium salmoninarum* infection in Atlantic salmon: a retrospective study of two sea ranches in Iceland. *Aquaculture* **186**, 193–203.

Gutenberger S.K., Giovannoni S.J., Field K.G., Fryer J.L. & Rohovec J.S. (1991) A phylogenetic comparison of the 16S rRNA sequence of the fish pathogen, *Renibacterium salmoninarum*, to Gram-positive bacteria. *FEMS Microbiology Letters* **77**, 151–156.

Gutenberger S.K., Duimstra J.R., Rohovec J.S. & Fryer J.L. (1997) Intracellular survival of *Renibacterium salmoninarum* in trout mononuclear phagocytes. *Disease of Aquatic Organisms* **28**, 93–106.

Halaihel N., Vendrell D., Ruiz-Zarzuela I., de Blas I., Alonso J.L., Gironés O., Pérez T. & Muzquiz J.L. (2009) A new real time PCR-based assay for diagnosing *Renibacterium salmoninarum* in rainbow trout (*Oncorhynchus mykiss*) and comparison with other techniques. *Journal of Microbiological Methods* **76**, 75–80.

Hallett S.L. & Bartholomew J.L. (2006) Application of a real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in river water samples. *Diseases of Aquatic Organisms* **71**, 109–118.

Hamel O.S. & Anderson J.J. (2002) Relationship between antigen concentration and bacterial load in Pacific salmon with bacterial kidney disease. *Diseases of Aquatic Organisms* **51**, 85–92.

Hendricks J.D. & Leek S.L. (1975) Kidney disease postorbital lesions in chinook salmon (*Oncorhynchus tshawytscha*). *Transactions of the American Fisheries Society* **104**, 805–807.

Hirvelä-Koski V., Pohjanvirta T., Koski P. & Sukura A. (2006) Atypical growth of *Renibacterium salmoninarum* in subclinical infections. *Journal of Fish Diseases* **29**, 21–29.

Hoffmann R., Popp W. & van de Graaff S. (1984) Atypical BKD predominantly causing ocular and skin lesions. *Bulletin of the European Association of Fish Pathologists* **4**, 7–9.

Jansson E., Hongslo T., Höglund J. & Ljungberg O. (1996) Comparative evaluation of bacterial culture and two ELISA techniques for the detection of *Renibacterium salmoninarum* antigens in salmonid kidney tissues. *Diseases of Aquatic Organisms* **27**, 197–206.

Jansson E., Lindberg L., Säker E. & Aspán A. (2008) Diagnosis of bacterial kidney disease by detection of *Renibacterium salmoninarum* by real-time PCR. *Journal of Fish Diseases* **31**, 755–763.

Jónsdóttir H., Malmquist H.J., Snorrason S.S., Gudbergsson G. & Gudmundsdóttir S. (1998) Epidemiology of *Renibacterium salmoninarum* in wild Arctic charr and brown trout in Iceland. *Journal of Fish Biology* **53**, 322–339.

Lemarchand K., Parthuisot N., Catala P. & Lebaron P. (2001) Comparative assessment of epifluorescence microscopy, flow cytometry and solid-phase cytometry used in the enumeration of specific bacteria in water. *Aquatic Microbial Ecology* **25**, 301–309.

Lisle J.T., Hamilton M.A., Willse A.R. & McFeters G.A. (2004) Comparison of fluorescence microscopy and solid-phase cytometry methods for counting bacteria in water. *Applied and Environmental Microbiology* **70**, 5343–5348.

Lovely J.E., Cabo C., Griffiths S.G. & Lynch W.H. (1994) Detection of *Renibacterium salmoninarum* infection in

asymptomatic Atlantic salmon. *Journal of Aquatic Animal Health* **6**, 126–132.

McKibben C.L. & Pascho R.J. (1999) Shedding of *Renibacterium salmoninarum* by infected chinook salmon *Oncorhynchus tschawytscha*. *Diseases of Aquatic Organisms* **38**, 75–79.

Metzger D.C., Elliott D.G., Wargo A., Park L.K. & Purcell M.K. (2010) Pathological and immunological responses associated with differential survival of Chinook salmon following *Renibacterium salmoninarum* challenge. *Diseases of Aquatic Organisms* **90**, 31–41.

Meyers T.R., Short S., Farrington C., Lipson K., Geiger H.J. & Gates R. (1993a) Comparison of the enzyme-linked immunosorbent assay (ELISA) and fluorescent antibody test (FAT) for measuring the prevalences and levels of *Renibacterium salmoninarum* in wild and hatchery stocks of salmonid fishes in Alaska, USA. *Diseases of Aquatic Organisms* **16**, 181–189.

Meyers T.R., Short S., Farrington C., Lipson K., Geiger H.J. & Gates R. (1993b) Establishment of a negative-positive threshold optical density value for the enzyme-linked immunosorbent assay (ELISA) to detect soluble antigen of *Renibacterium salmoninarum* in Alaskan Pacific salmon. *Diseases of Aquatic Organisms* **16**, 191–197.

Meyers T.R., Korn D., Glass K., Burton T., Short S., Lipson K. & Starkey N. (2003) Retrospective analysis of antigen prevalences of *Renibacterium salmoninarum* (Rs) detected by enzyme-linked immunosorbent assay in Alaskan Pacific salmon and trout from 1988 to 2000 and management of Rs in hatchery Chinook and coho salmon. *Journal of Aquatic Animal Health* **15**, 101–110.

Miriam A., Griffiths S.G., Lovely J.E. & Lynch W.H. (1997) PCR and probe-PCR assays to monitor broodstock Atlantic salmon (*Salmo salar* L.) ovarian fluid and kidney tissue for presence of DNA of the fish pathogen *Renibacterium salmoninarum*. *Journal of Clinical Microbiology* **35**, 1322–1326.

Mitchum D.L. & Sherman L.E. (1981) Transmission of bacterial kidney disease from wild to stocked hatchery trout. *Canadian Journal of Fisheries and Aquatic Sciences* **38**, 547–551.

Munson A.D., Elliott D.G. & Johnson K. (2010) Management of bacterial kidney disease in Chinook salmon hatcheries based on broodstock testing by enzyme-linked immunosorbent assay: a multiyear study. *North American Journal of Fisheries Management* **30**, 940–955.

Murray C.B., Evelyn T.P.T., Beacham T.D., Barner L.W., Ketcheson J.E. & Prosperi-Porta L. (1992) Experimental induction of bacterial kidney disease in chinook salmon by immersion and cohabitation challenges. *Disease of Aquatic Organisms* **12**, 91–96.

Nance S.L., Riederer M., Zubkowski T., Trudel M. & Rhodes L.D. (2010) Interpreting dual ELISA and qPCR data for bacterial kidney disease of salmonids. *Diseases of Aquatic Organisms* **91**, 113–119.

O'Farrell C.L. & Strom M.S. (1999) Differential expression of the virulence-associated protein p57 and characterization of its duplicated gene msa in virulent and attenuated strains of *Renibacterium salmoninarum*. *Diseases of Aquatic Organisms* **38**, 115–123.

O'Farrell C.L., Elliott D.G. & Landolt M.L. (2000) Mortality and kidney histopathology of chinook salmon *Oncorhynchus tshawytscha* exposed to virulent and attenuated *Renibacterium salmoninarum* strains. *Diseases of Aquatic Organisms* **43**, 199–209.

OIE (2003) *Manual of Diagnostic Tests for Aquatic Animals*, 4th edn. Office International des Épizooties, Paris.

OIE (2009) *Manual of Diagnostic Tests for Aquatic Animals*, 6th edn. Office International des Épizooties, Paris.

Olsen A.B., Hopp P., Binde M. & Grønstøl H. (1992) Practical aspects of bacterial culture for the diagnosis of bacterial kidney disease. *Disease of Aquatic Organisms* **14**, 207–212.

Ordal E.J. & Earp B.J. (1956) Cultivation and transmission of the etiological agent of kidney disease in salmonid fishes. *Proceedings of the Society for Experimental Biology and Medicine* **92**, 85–88.

Paclibare J.O., Evelyn T.P.T. & Albright L.J. (1988) A comparative evaluationof various methods for detection of the kidney disease bacterium *Renibacterium salmoninarum* in salmonids. *Bulletin of the Aquaculture Association of Canada* **88**, 110–112.

Pascho R.J., Elliott D.G., Mallett R.W. & Mulcahy D. (1987) Comparison of five techniques for the detection of *Renibacterium salmoninarum* in adult coho salmon. *Transactions of the American Fisheries Society* **116**, 882–890.

Pascho R.J., Elliott D.G. & Streufert J.M. (1991) Brood stock segregation of spring chinook salmon *Oncorhynchus tshawytscha* by use of the enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody technique (FAT) affects the prevalence and levels of *Renibacterium salmoninarum* infection in progeny. *Diseases of Aquatic Organisms* **12**, 25–40.

Pascho R.J., Goodrich T.D. & McKibben C.L. (1997) Evaluation by enzyme-linked immunosorbent assay (ELISA) of *Renibacterium salmoninarum* bacterins affected by persistence of bacterial antigens. *Journal of Aquatic Animal Health* **9**, 99–107.

Pascho R.J., Chase D. & McKibben C.L. (1998) Comparison of the membrane-filtration fluorescent antibody test, the enzyme-linked immunosorbent assay, and the polymerase chain reaction to detect *Renibacterium salmoninarum* in salmonid ovarian fluid. *Journal of Veterinary Diagnostic Investigation* **10**, 60–66.

Pascho R.J., Elliott D.G. & Chase D.M. (2002) Comparison of traditional and molecular methods for detection of *Renibacterium salmoninarum*. In: *Molecular Diagnosis of Salmonid Diseases* (ed. by C.O. Cunningham), pp. 157–209. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Pfaffl M.W. (2004) Quantification strategies in real-time PCR. In: *A–Z of Quantitative PCR* (ed. by S.A. Bustin), pp. 87–120. International University Line, La Jolla, CA.

Powell M., Overturf K., Hogge C. & Johnson K. (2005) Detection of *Renibacterium salmoninarum* in chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), using quantitative PCR. *Journal of Fish Diseases* **28**, 615–622.

Purcell M.K., Murray A.L., Elz A., Park L.K., Marcquenski S.V., Winton J.R., Alcorn S.W., Pascho R.J. & Elliott D.G. (2008) Decreased mortality of Lake Michigan Chinook

Published 2013.

salmon after bacterial kidney disease challenge: evidence for pathogen-driven selection? *Journal of Aquatic Animal Health* **20**, 225–235.

Purcell M.K., Getchell R.G., McClure C.A. & Garver K.A. (2011) Quantitative polymerase chain reaction (PCR) for detection of aquatic animal pathogens in a diagnostic laboratory setting. *Journal of Aquatic Animal Health* **23**, 148–161.

Rauhut R. & Klug G. (1999) mRNA degradation in bacteria. *FEMS Microbiology Reviews* **23**, 353–370.

Rhodes L.D., Coady A.M. & Deinhard R.K. (2004) Identification of a third *msa* gene in *Renibacterium salmoninarum* and the associated virulence phenotype. *Applied and Environmental Microbiology* **70**, 6488–6494.

Rhodes L.D., Durkin C., Nance S.L. & Rice C.A. (2006) Prevalence and analysis of *Renibacterium salmoninarum* infection among juvenile Chinook salmon *Oncorhynchus tshawytscha* in North Puget Sound. *Diseases of Aquatic Organisms* **71**, 179–190.

Sakai M. & Kobayashi M. (1992) Detection of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonid fish, from pen-cultured coho salmon. *Applied and Environmental Microbiology* **58**, 1061–1063.

Sakai M., Koyama G., Atsuta S. & Kobayashi M. (1987) Detection of *Renibacterium salmoninarum* by a modified peroxidase-antiperoxidase (PAP) procedure. *Fish Pathology* **22**, 1–5.

Sambrook S., Fritsch E.F. & Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Springs Harbor Laboratory, New York.

Sandell T.A. & Jacobson K.C. (2011) Comparison and evaluation of *Renibacterium salmoninarum* quantitative PCR diagnostic assays using field samples of Chinook and coho salmon. *Diaseases of Aquatic Organisms* **93**, 129–139.

Sanders J.E., Pilcher K.S. & Fryer J.L. (1978) Relation of water temperature to bacterial kidney disease in coho salmon (*Oncorhynchus kisutch*), sockeye salmon (*Oncorhynchus nerka*) and steelhead trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board of Canada* **35**, 8–11.

Senson P.R. & Stevenson R.M.W. (1999) Production of the 57 kDa major surface antigen by a non-agglutinating strain of the fish pathogen *Renibacterium salmoninarum*. *Diseases of Aquatic Organisms* **38**, 23–31.

Sheridan G.E.C., Masters C.I., Shallcross J.A. & Mackey B.M. (1998) Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. *Applied and Environmental Microbiology* **64**, 1313–1318.

Smith R.D. (2006) *Veterinary Clinical Epidemiology*, 3rd edn. Taylor and Francis, Boca Raton, FL.

Speare D.J. (1997) Differences in patterns of meningoencephalitis due to bacterial kidney disease in farmed Atlantic and chinook salmon. *Research in Veterinary Science* **62**, 79–80.

Stackebrandt E., Wehmeyer U., Nader H. & Fiedler F. (1988) Phylogenetic relationship of the fish pathogenic *Renibacterium salmoninarum* to *Arthrobacter*, *Micrococcus*, and related taxa. *FEMS Microbiology Letters* **50**, 117–120.

Starliper C.E. (1996) Genetic diversity of North American isolates of *Renibacterium salmoninarum*. *Diseases of Aquatic Organisms* **27**, 207–213.

Starliper C.E. & Teska J.D. (1995) Relevance of *Renibacterium salmoninarum* in an asymptomatic carrier population of brook trout, *Salvelinus fontinalis* (Mitchill). *Journal of Fish Diseases* **18**, 383–387.

Suzuki K. & Sakai D.K. (2007) Real-time PCR for quantification of viable *Renibacterium salmoninarum* in chum salmon *Oncorhynchus keta*. *Diseases of Aquatic Organisms* **74**, 209–223.

Teska J.D. (1993) *In vitro* growth enhancement of *Renibacterium salmoninarum*: the aetiology of bacterial kidney disease. *Biomedical Letters* **48**, 27–33.

Teska J.D., Dawson A., Starliper C.E. & Tillinghast D. (1995) A multiple-technique approach to investigating the presumptive low-level detection of *Renibacterium salmoninarum* at a broodstock hatchery in Maine. *Journal of Aquatic Animal Health* **7**, 251–256.

Thrusfield M. (2005) *Veterinary Epidemiology*, 3rd edn. Blackwell Science Ltd., Oxford, UK.

Turaga P.S.D., Wiens G.D. & Kaattari S.L. (1987) Bacterial kidney disease: the potential role of soluble protein antigen(s). *Journal of Fish Biology* **31**(Suppl. A), 191–194.

Westgard J.O. (2008) *Basic Method Validation*, 3rd edn. Westgard QC, Madison, WI.

Wiens G.D. & Kaattari S.L. (1989) Monoclonal antibody analysis of common surface protein(s) of *Renibacterium salmoninarum*. *Fish Pathology* **24**, 1–7.

Wiens G.D., Pascho R. & Winton J.R. (2002) A single Ala^{139}-to-Glu substitution in the *Renibacterium salmoninarum* virulence-associated protein p57 results in antigenic variation and is associated with enhanced p57 binding to chinook salmon leukocytes. *Applied and Environmental Microbiology* **68**, 3969–3977.

Wiens G.D., Rockey D.D., Wu Z., Chang J., Levy R., Crane S., Chen D.S., Capri G.R., Burnett J.R., Sudheesh P.S., Schipma M.J., Burd H., Bhattacharyya A., Rhodes L.D., Kaul R. & Strom M.S. (2008) Genome sequence of the fish pathogen *Renibacterium salmoninarum* suggests reductive evolution away from an environmental *Arthrobacter* ancestor. *Journal of Bacteriology* **190**, 6970–6982.

Yoshimizu M., Ji R. & Kimura T. (1987) A falsepositive [sic] reaction in the indirect fluorescent antibody test for *Renibacterium salmoninarum* ATCC 33209 caused by *Pseudomonas* sp. *Scientific Reports of the Hokkaido Fish Hatchery* **41**, 121–127.

Young C.L. & Chapman G.B. (1978) Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (*Salvelinus fontinalis)*. *Journal of the Fisheries Research Board of Canada* **35**, 1234–1248.

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