

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

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ABSTRACT: As part of a screening program to reduce the vertical transmission of bacterial kidney disease (BKD) in 1992, kidney tissue and ovarian fluid from subclinically affected broodstock Atlantic salmon *Salmo salar* L. were assayed for the presence of *Renibacterium salmoninarum*. Of the methods used, culture on selective kidney disease medium (SKDM) agar identified the highest number of samples positive for *R. salmoninarum* followed by the indirect fluorescent antibody technique (IFAT). The comparatively low sensitivities of an enzyme-linked immunosorbent assay (ELISA) and Western blot assay reflected the low levels of *R. salmoninarum* in most infected samples. Also, for many individual fish sampled, *R. salmoninarum* was detected either in the kidney tissue or in ovarian fluid but not in both. In an attempt to increase the level of detection, a method involving incubation of ovarian fluid cellular debris in SKDM broth followed by Western blotting was used to screen ovarian fluids in 1993. This relatively simple procedure identified more positive samples than either of the established methods of SKDM agar culture or IFAT. Although it increased the total numbers of positive samples identified by 32%, incomplete correlation among methods was observed. Therefore, to ensure that the maximum number of positive samples is detected, it is advisable to use a combination of these methods.

KEY WORDS: BKD · Detection · Atlantic salmon

INTRODUCTION

Bacterial kidney disease (BKD) caused by the Gram-positive bacterium *Renibacterium salmoninarum* continues to be a problem for salmonid aquaculture on an international basis (Evenden et al. 1993). In eastern Canada, the disease affects chiefly 2 and 3 yr sea-raised Atlantic salmon *Salmo salar* L. It is not that uncommon to identify infection in 30% or more of market or broodstock fish by culture from kidney tissue, the majority of which show no obvious external or internal clinical signs of disease (Lovely et al. 1994). The local experience is that BKD usually causes a low-level mortality although serious losses may occur

over short periods. The affected individuals generally exhibit little if any external clinical signs, but upon internal examination the pathology may include grossly convex greyish kidney tissue and white granulomatous lesions in all major internal organs.

Due to lack of effective chemotherapy and the absence of a vaccine, screening of reproductive fluids from broodstock for *Renibacterium salmoninarum* is considered to be the most effective means of avoiding transmission of the pathogen. The ovarian fluid surrounding eggs from female fish is collected for this purpose. *R. salmoninarum* cells present in ovarian fluids are able to passively infect eggs intra ovum (Evelyn et al. 1984, 1986, Lee & Evelyn 1989). In con-

trast, the reproductive fluid of male broodstock is not believed to have a significant effect on the vertical transmission of BKD, even when the milt is heavily contaminated (Evelyn et al. 1986).

The indirect fluorescent antibody technique (IFAT) (Bullock & Stuckey 1975) and culture on selective kidney disease medium (SKDM) agar (Austin et al. 1983) are the most common methods for screening ovarian fluids in eastern Canada. However, IFAT is regarded as a relatively insensitive method (unless combined with membrane filtration; Elliot & Barila 1987) when compared to culture on SKDM agar (Armstrong et al. 1989). Furthermore, whereas culture is the more relevant and sensitive method for the detection of viable *Renibacterium salmoninarum*, incubation for approximately 6 wk is required before individual colonies can be observed. Other authors report that incubation for up to 19 wk may be required for the successful culture of some isolates (Benediktsdottir et al. 1991).

In the first part of our study, conducted in 1992, kidney tissue and ovarian fluid from broodstock Atlantic salmon showing no signs of BKD were assayed by various methods to detect *Renibacterium salmoninarum* [SKDM agar culture, IFAT, an enzyme-linked immunosorbent assay (ELISA), and Western blotting]. The initial purpose was to compare the methods for their ability to detect the pathogen in subclinically infected broodstock. Because negative ovarian fluid results were obtained for a high percentage of fish that proved positive by culture of kidney tissue, we suspected that some ovarian fluids containing low levels of *R. salmoninarum* had escaped detection. Therefore, a method combining growth enrichment in SKDM broth with Western blotting was incorporated into a subsequent screening of ovarian fluids in 1993 in an effort to confirm this suspicion. The hypothesis was that small numbers of *R. salmoninarum* might be present in concentrated ovarian fluid cellular debris which could be incubated in SKDM broth. This might permit growth of the pathogen to a level where Western blots of the cultured material would reveal the presence of a diagnostic marker antigen (the p57 protein; Wiens & Kaattari 1991) for *R. salmoninarum*. The results obtained using this method were compared to those obtained with established methods of SKDM agar culture and IFAT.

MATERIALS AND METHODS

Collection of ovarian fluid and kidney tissue. Ovarian fluid samples were obtained in November 1992 from 200 gravid female Atlantic salmon held at a farm (Farm D) in the Bay of Fundy, New Brunswick,

Canada. Under field conditions, eggs and ovarian fluid from individual fish were collected by industry personnel in clean, non-sterile containers. Ovarian fluid samples were then immediately collected from below the surface of the liquid using 3 ml sterile vacutainer tubes with needle units (Becton Dickinson Co., Rutherford, NJ, USA). The samples were stored overnight at 4°C prior to processing.

Ovarian fluid samples (1.5 ml) were sedimented at 2000 × *g* for 10 min in a centrifuge (Model HN, International Equipment Co., Needham Hts., MA, USA) and the pellet washed with 1.5 ml phosphate buffered saline (pH 7.2). After decanting, the pellet was suspended in the fluid (approximately 50 to 100 µl) remaining in the centrifuge tube and 5 µl was applied to glass slides, fixed with acetone, and stained for IFAT (Bullock & Stuckey 1975) using a polyclonal antiserum prepared in this laboratory (Griffiths & Lynch 1991). A sterile cotton swab was inserted into the suspended pellet and used to spread the sample over the surface of SKDM agar plates (Austin et al. 1983). The SKDM agar plates were incubated at 15°C for 6 wk, and growth appearing was confirmed as *Renibacterium salmoninarum* by Western blotting (Griffiths et al. 1991, Lovely et al. 1994). Aliquots of the (non-centrifuged) ovarian fluid samples were assayed by an ELISA (K-Dtect) as per the manufacturer's instructions (DiagXotics Inc., Wilton, CT, USA).

Kidneys were removed from the above fish, stored at 4°C overnight, and homogenized as previously reported (Olivier et al. 1992). Resulting homogenates were assayed for *Renibacterium salmoninarum* by the IFAT, by culture on SKDM agar, by ELISA as above, and by the Western blot assay. The polyclonal antiserum, previously described, was used for the IFAT and the Western blot assay.

Analysis for *Renibacterium salmoninarum* in ovarian fluid samples. Ovarian fluid samples (total of 601) were collected in November 1993 from gravid female Atlantic salmon held at 6 separate farms in the Bay of Fundy and subjected to analysis by IFAT and culture on SKDM agar as described above.

In addition, the ovarian fluid samples were screened by a growth enrichment step followed by Western blotting, as described below. Separate aliquots (1.5 ml) of each ovarian fluid sample were sedimented as described previously and the supernatants decanted. The pellets were suspended in 3 ml of SKDM broth and incubated at 15°C for 3 wk without agitation. The broths were then sedimented, and the pellets were suspended in 200 µl of electrophoresis sample buffer (Laemmli 1970) and placed in a boiling water bath for 3 min. The samples were sedimented at 14 000 × *g* for 5 min in a microfuge,

and 5 µl aliquots of the supernatants were subjected to SDS-PAGE and transferred to nitrocellulose paper as previously reported (Griffiths et al. 1991). Samples of 50 ng (by dry wt) of extracellular products harvested from laboratory cultures of *Renibacterium salmoninarum* (Griffiths & Lynch 1991) and pre-stained molecular mass markers (Bio-Rad Laboratories Ltd., Hercules, CA, USA) were included on each SDS-PAGE gel.

For the Western blots using a monoclonal antibody, the nitrocellulose papers were washed with tris buffered saline (10 mM Tris-HCl, 250 mM NaCl, pH 7.5) containing 0.05% v/v Tween-20 (TTBS) for 30 min. All subsequent binding agent steps were conducted in a total volume of 10 ml of TTBS. The first binding agent was a 5×10^{-2} dilution of ascites fluid containing a monoclonal antibody reactive with the *Renibacterium salmoninarum* p57 antigen (kindly provided by F. Markham, Atlantic Veterinary College, Charlottetown, PEI, Canada) (60 min incubation), the second was 5×10^{-3} dilution of biotinylated goat anti-mouse immunoglobulin (Pierce, Rockford, IL, USA) (30 min incubation), and the third was a 3×10^{-3} dilution of streptavidin alkaline phosphatase (Amersham Corp., Arlington Heights, IL) (15 min incubation). Each binding agent step was followed by 2 washes with TTBS for 5 min. Colour development of the blots was as previously reported (Griffiths et al. 1991). All incubations and washes were performed using a Western roller TM, Model 106 (Biocomp Instruments Inc., Frederick, NB).

RESULTS

Detection of *Renibacterium salmoninarum* in kidney tissue and ovarian fluid

In 1992, culture from kidney tissue identified the highest number of female Atlantic salmon broodstock infected with *Renibacterium salmoninarum* (34.5%; Table 1). Other methods used to analyze kidney tissue identified lower numbers of infected fish: 12% by IFAT, 4% by ELISA, and 1% by the Western blot assay. Analysis of ovarian fluid samples collected from the same fish produced 19.5% positive by culture, 7.5% positive by IFAT and 0% positive by ELISA. (Due to the low sensitivity of the Western blot assay only 35 ovarian fluid samples were tested and all were negative.)

The correlations between the BKD detection methods and between detection of *Renibacterium salmoninarum* in kidney tissue and ovarian fluids are shown in Table 1. A total of 91 of the 200 fish tested positive where the results for all of the assay methods on kidney tissue and ovarian fluid were considered. Of these 91 fish each method identified the following: 76% by kidney culture, 43% by ovarian fluid culture, 26% by kidney IFAT, 14% by ovarian fluid IFAT, 9% by kidney ELISA, 2% by kidney Western blot, and 0% by ovarian fluid ELISA. Interestingly, 37 of these 91 fish were identified only by kidney culture, 15 were identified only by ovarian fluid culture, 3 were identified only by kidney IFAT, and 2 were identified only by ovarian fluid IFAT.

Table 1 Comparison of methods to detect *Renibacterium salmoninarum* in ovarian fluids and kidney tissue from broodstock Atlantic salmon collected in 1992

No. of fish sampled	Kidney				Ovarian fluid		
	Culture on SKDM agar	IFAT technique	ELISA	Western blot assay	Culture on SKDM agar	IFAT technique	ELISA
109	-	-	-	-	-	-	-
37	+	-	-	-	-	-	-
15	-	-	-	-	+	-	-
8	+	-	-	-	+	-	-
5	+	+	-	-	-	-	-
3	+	+	+	-	-	-	-
3	+	+	-	-	+	+	-
3	+	+	-	-	+	-	-
3	-	+	-	-	-	-	-
3	+	-	-	-	+	+	-
2	+	+	+	+	+	+	-
2	+	+	+	-	+	-	-
2	+	-	-	-	-	+	-
2	-	+	-	-	+	+	-
2	-	-	-	-	-	+	-
1	+	+	+	-	+	+	-
Totals 200	69 (34.5%)	24 (12%)	8 (4%)	2 (1%)	39 (19.5%)	15 (7.5%)	0 (0%)

It is possible that not all subclinically infected female fish produced ovarian fluids containing viable *Renibacterium salmoninarum* (i.e. bacteria may have been present in one tissue sample but not the other) because 47 of the 69 fish positive by kidney culture produced ovarian fluid that yielded negative results by culture. However, 17 of the 39 culture-positive ovarian fluids were collected from fish whose kidneys produced negative results by culture. We suspected that a significant number of ovarian fluids containing low levels of *R. salmoninarum* may not have been identified by the methods used.

Growth enrichment in SKDM broth combined with Western blotting

An additional method involving culture of ovarian fluid cellular debris in SKDM broth followed by Western blotting of the cultured material was used to screen ovarian fluid samples collected in 1993.

Positive ovarian fluid samples, following incubation in SKDM broth, were rapidly identified by Western blotting which detected a major immunoreactive band (Fig. 1, lanes 1, 4 and 6) corresponding to the p57 protein of *R. salmoninarum* (Fig. 1, lane 12). Despite the use of a monoclonal antibody reactive with p57, other bands from the ovarian fluid samples were found to be reactive at varying degrees of intensity in the Western blot assay. These bands even occurred in the absence of a detectable p57 band (Fig. 1, lanes 2, 3, 5, 7-9; and Fig. 2A, lanes 2, 4-6). These reactive bands appeared to be due to non-specific cross-reaction of ovarian fluid

proteins and/or SKDM components with the secondary biotinylated antibody and streptavidin alkaline phosphatase because, unlike the p57 band, they tended to occur even when the primary p57 monoclonal antibody was omitted from the Western blot assay (Fig. 2B). These non-specific reactions did not pose any hindrance to the interpretation of the Western blot results. Positive ovarian fluid samples contained insufficient p57 antigen to be detected prior to their incubation in SKDM broth (Fig. 2A).

Multiple bands (other than p57) observed in the extracellular products harvested from *Renibacterium salmoninarum* SKDM agar cultures (Fig. 1, lane 12) were due to reaction of the monoclonal antibody with proteolytic degradation products and complexes of p57 that form in concentrated extracellular products (Griffiths & Lynch 1991, Rockey et al. 1991, Wiens & Kaatari 1991).

Attempts to isolate and identify *Renibacterium salmoninarum* colony-forming units on SKDM agar subsequent to incubation of the ovarian fluid samples (both positive and negative) in SKDM broth were precluded by the presence of other faster growing microorganisms which presumably had increased in numbers during incubation in the SKDM broth.

Comparison of the growth enrichment/Western blot assay with culture on SKDM agar and IFAT

Culture on SKDM agar and IFAT were used as the established methods to screen 601 ovarian fluid samples (kidney samples were not made available)

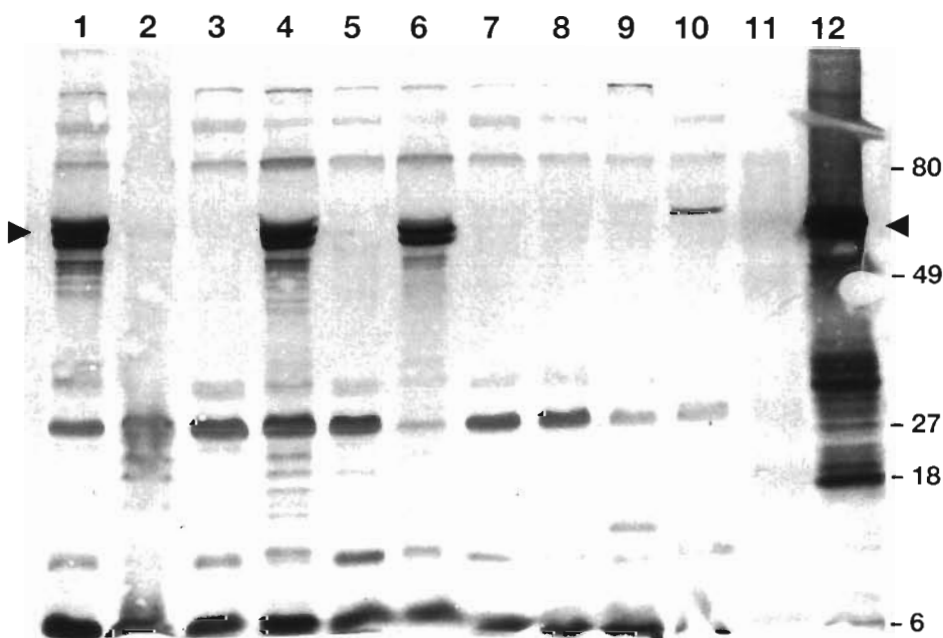


Fig. 1. Western blot detection of the *Renibacterium salmoninarum* major soluble antigen (indicated by arrowheads) following incubation of ovarian fluid cellular debris in selective kidney disease broth medium. Ovarian fluid samples positive (lanes 1, 4, and 6), weakly positive (lane 10), and negative (lanes 2, 3, 5, and 7-9) for the *R. salmoninarum* major antigen. Pre-stained molecular mass markers are shown in lane 11 and extracellular products from a laboratory culture of *R. salmoninarum* are shown in lane 12

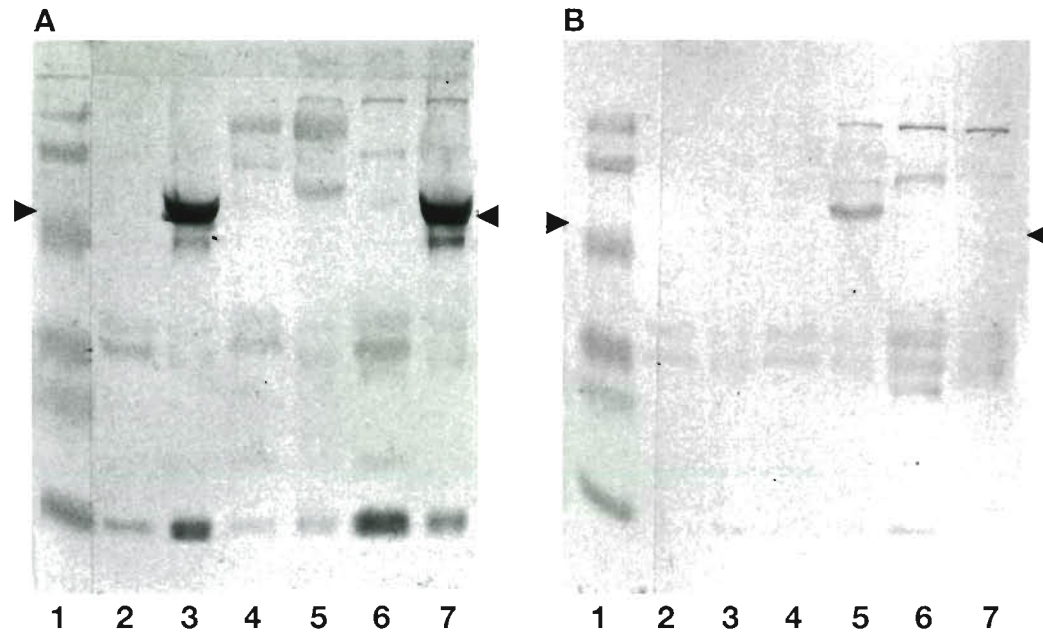


Fig. 2. (A) Western blot detection of the *Renibacterium salmoninarum* major soluble antigen (indicated by arrowheads) in ovarian fluid cellular debris samples before (lanes 2, 4, and 6) and after incubation of the same samples in selective kidney disease broth medium (lanes 3, 5, and 7). Incubated ovarian fluid samples positive (lanes 3 and 7) and negative (lane 5) for the *R. salmoninarum* major antigen. (B) Same samples as in (A) except that the monoclonal anti-p57 antibodies were omitted from the Western blot assay. Lanes 1 of (A) and (B) contained pre-stained molecular mass standards

obtained from 6 farms in the Bay of Fundy in 1993 and the results obtained were compared to those obtained using growth enrichment in SKDM broth followed by Western blotting (Table 2). The number of ovarian fluids positive for *Renibacterium salmoninarum* by the methods tested ranged from 0 to 36.6%, depending on the farm from which the samples were collected. A total of 75 of the 601 samples tested positive. Of these positive samples, more were identified by a combination of growth enrichment and Western blotting (50) than by culture on SKDM agar (39) or IFAT (36).

A comparison between the methods (Table 3) showed that only 19% of the positive ovarian fluid samples produced a positive result by all 3 methods

and only 48% of the positive samples gave a positive result by 2 or more of the methods. These results emphasized the inadequacy of any single method for identifying all ovarian fluid samples containing *Renibacterium salmoninarum*.

DISCUSSION

A high prevalence (34.5%) of subclinical BKD infection was detected in 200 post-spawned female broodstock Atlantic salmon in 1992 by culture of *Renibacterium salmoninarum* from kidney tissue samples on SKDM agar. However, for many of the individual fish,

Table 2. Detection of *Renibacterium salmoninarum* in ovarian fluid samples collected in 1992 from broodstock Atlantic salmon from 6 farms

Farm site	No. of samples	No. of samples positive			Percentage of samples positive by the methods tested
		IFAT technique	Culture on SKDM agar	Growth enrichment with Western blot assay	
A	60	0	0	0	0
B	90	1	2	5	6.7
C	169	0	0	6	3.6
D	139	13	17	24	19.3
E	60	4	0	2	10
F	82	18	20	13	36.6
Totals	601	36	39	50	12.5

Table 3. Comparison of methods to detect *Renibacterium salmoninarum* in ovarian fluid samples from broodstock Atlantic salmon collected in 1992

No. of samples	IFAT technique	Culture on SKDM agar	Growth enrichment with Western blot assay
526	-	-	-
18	-	-	+
14	+	+	+
14	-	+	+
14	+	-	-
7	-	+	-
4	+	+	-
4	+	-	+
Totals 601	36	39	50

R. salmoninarum was cultivated from only 1 of the 2 tissues tested (kidney tissue or ovarian fluid). These disparities between kidney and ovarian fluid from the same fish may relate to the low levels of viable *R. salmoninarum* cells in tissues of many of the subclinically infected fish and to the difficulties associated with their successful culture from such tissues (Evelyn 1977, Daly & Stevenson 1988, Evelyn & Prospero-Porta 1989).

IFAT identified fewer of the infected tissue samples than culture on SKDM agar using either kidney or ovarian fluid samples; this was consistent with earlier observations by Armstrong et al. (1989) and Griffiths et al. (1991). However, a few IFAT-positive, culture-negative tissue samples were recorded. It is possible that these results were due to tissue samples containing *Renibacterium salmoninarum* cells that were non-viable or in a form unable to grow on the SKDM agar under the conditions used. Another possibility is that the cell shapes identified by IFAT were other bacteria which cross-reacted with the polyclonal antiserum used (Austin et al. 1985, Yoshimura et al. 1987, Foott et al. 1992). The low sensitivities of *R. salmoninarum* detection using the ELISA or the Western blot assay, even when compared to IFAT, suggested that the amount of soluble antigen present in many samples was very low. This observation further suggests that low numbers of relatively inactive *R. salmoninarum* cells occurred in the kidney tissue and ovarian fluid of most of the subclinically infected fish tested (Lovely et al. 1994).

In 1993, the cultivation of ovarian fluids in SKDM broth followed by Western blotting detected more samples positive for *Renibacterium salmoninarum* than either SKDM agar culture or IFAT and increased the total number of positive samples by 32%. These results suggested that a significant number of ovarian fluids containing live pathogen escaped detection by the established methods.

Incomplete correlation between the 3 methods was observed, but most of the ovarian fluids scored positive

by any one method (89, 64, and 61%, respectively, for SKDM agar culture, culture in SKDM broth with Western blotting and IFAT) also were scored positive by at least one other method. For immunoassays (IFAT, ELISA or Western blot assay), false-positive reactions caused by cross-reactions with antigens other than those from *Renibacterium salmoninarum* are possible (Austin et al. 1985, Yoshimura et al. 1987, Foott et al. 1992, Bandin et al. 1993, Brown et al. 1994). However, with the use of the monoclonal antibody to detect and positionally locate the *R. salmoninarum* p57 antigen on Western blots the possibility of false-positives

should be minimized. On the other hand, several SKDM agar culture-positive ovarian fluids were scored negative by the SKDM broth culture/Western blot technique. It is possible that some ovarian fluid samples contained very few *R. salmoninarum* cells and that not all aliquots from the same sample contained live pathogen. Other factors influencing pathogen growth may have included unidentified inhibitory components present in the ovarian fluid cellular debris and/or produced during the growth of other microorganisms (Evelyn 1977, Daly & Stevenson 1988, Evelyn & Prospero-Porta 1989). Difficulties with the culture of live pathogen may indicate that ovarian fluids scoring positive by IFAT but negative by the culture may still contain live *R. salmoninarum*.

In the local management of BKD screening programs a 'zero tolerance' attitude is often adopted as standard policy (S. Roach, General Manager, Stolt Sea Farm, Saint John, NB, pers. comm.). Our results indicate that for screening it would be most advantageous to use a combination of methods (eg. SKDM broth culture with Western blot, SKDM agar culture, and IFAT) to increase the number of ovarian fluid samples containing *Renibacterium salmoninarum* that are identified. Future assays for BKD monitoring will probably involve the targeting of specific stretches of *R. salmoninarum* DNA using the polymerase chain reaction (PCR) (Brown et al. 1994, Leon et al. 1994), and it is possible these assays will provide a more sensitive method for detecting *R. salmoninarum*.

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