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Comparison of two fluorescent antibody techniques (FATs) for detection and quantification of *Renibacterium salmoninarum* in coelomic fluid of spawning chinook salmon *Oncorhynchus tshawytscha*

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ABSTRACT: Two versions of the fluorescent antibody technique (FAT) were compared for detection and quantification of *Renibacterium salmoninarum* in coelomic fluid samples from naturally infected spawning chinook salmon *Oncorhynchus tshawytscha*. For the membrane filtration-FAT (MF-FAT), trypsin-treated samples were passed through 0.2 µm polycarbonate filters to concentrate bacteria for direct enumeration by immunofluorescence microscopy. For the smear-FAT (S-FAT), samples were centrifuged at 8800 × *g* for 10 min and the pelleted material was smeared on slides for immunofluorescence staining. Detected prevalences of *Renibacterium salmoninarum* were 1.8 to 3.4 times higher by the MF-FAT than by the S-FAT; differences were significant at $p \leq 0.0002$. The S-FAT consistently detected *R. salmoninarum* only in samples with calculated bacterial concentrations $\geq 2.4 \times 10^3$ cells ml^{-1} by MF-FAT testing. Increasing the area examined on a filter or slide from 50 to 100 microscope fields at 1000× magnification resulted in the detection of a maximum of 4% additional positive samples by the MF-FAT and 7% additional positive samples by the S-FAT. In individual samples for which bacterial counts were obtained by both the MF-FAT and the S-FAT, the counts averaged from 47 times (±30 SD) to 175 times (±165 SD) higher by the MF-FAT. Centrifugation of samples at 10000 × *g* for 10 min resulted in a 4-fold increase in mean bacterial counts by the S-FAT compared with a 10-min centrifugation at 2000 × *g*, but the highest calculated bacterial concentration obtained by S-FAT testing was more than 6-fold lower than that obtained for the same sample by MF-FAT testing. Because of its greater sensitivity, the MF-FAT is preferable to the S-FAT for use in critical situations requiring the detection of low numbers of *R. salmoninarum*.

KEY WORDS: *Renibacterium salmoninarum* · Broodstock screening · Fluorescent antibody technique (FAT) · BKD detection

INTRODUCTION

Bacterial kidney disease (BKD) is a significant obstacle to the successful artificial propagation of salmonids, particularly Pacific salmon *Oncorhynchus* spp. (Fryer & Lannan 1993). Control of this chronic systemic disease is complicated by the dual modes of transmission of the causative agent *Renibacterium salmoninarum*, which can be transmitted both vertically (Evelyn et al. 1984, 1986) and horizontally (Mitchum & Sherman 1981, Bell et al. 1984). Evidence suggests a positive correlation between the severity of infection in the female parent and the frequency and severity of infection in the progeny (Pascho et al. 1991), but the processes of vertical transmission of *R. salmoninarum* are not understood. Although some *R. salmoninarum* cells may become incorporated into developing oogonia (Bruno & Munro 1986), transmission also may occur by contact of the eggs with contaminated coelomic (ovarian) fluid (Evelyn et al. 1986, Lee & Evelyn 1989).

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Increased knowledge of the mechanisms of vertical transmission of *R. salmoninarum* is necessary for the development of more effective methods for preventing its occurrence.

Several rapid immunological methods have been used for detection of *Renibacterium salmoninarum* in tissues and body fluids of spawning fish. Such tests include the enzyme-linked immunosorbent assay (ELISA; Pascho et al. 1987, 1991), the membrane filtration-fluorescent antibody technique (MF-FAT; Elliott & Barila 1987, Pascho et al. 1987, 1991), and the smear FAT (S-FAT; Lee & Gordon 1987a, Armstrong et al. 1989). The ELISA detects soluble antigens produced by *R. salmoninarum*, whereas the FAT enables the enumeration of bacterial cells in samples. The ELISA can be used for rapid testing of samples from large numbers of fish, but the results of one study indicated that this test consistently detected the presence of *R. salmoninarum* antigen only in coelomic fluid samples with bacterial counts $>1 \times 10^5$ ml^{-1} as determined by the MF-FAT (Pascho et al. 1991). *Renibacterium salmoninarum* concentrations $<1 \times 10^2$ bacteria ml^{-1} can be detected in coelomic fluid by the MF-FAT (Elliott & Barila 1987). However, this procedure, which involves the concentration of *R. salmoninarum* from individual samples on membrane filters for direct enumeration by immunofluorescence microscopy, can be labour-intensive. The S-FAT, a procedure in which samples are centrifuged and the pelleted material is smeared on slides for immunofluorescence staining, can be performed more rapidly than the MF-FAT, but may be less sensitive. Lee & Gordon (1987b) reported higher *R. salmoninarum* detection rates in coelomic fluid samples tested by the MF-FAT compared with the S-FAT, but they did not describe the procedures used for processing and analyzing samples for the S-FAT.

The purpose of our study was to determine the relative sensitivities of the MF-FAT and the S-FAT for the detection of *Renibacterium salmoninarum* in the coelomic fluid of naturally infected chinook salmon *Oncorhynchus tshawytscha*. The effect of centrifugation speed on the sensitivity of the S-FAT was also investigated.

MATERIALS AND METHODS

Coelomic fluid samples. Coelomic fluid samples were obtained from spawning spring chinook salmon at Dworshak National Fish Hatchery (NFH), U.S. Fish and Wildlife Service (USFWS), Ahsahka, Idaho, USA, in 1988 and at Carson NFH (USFWS), Carson, Washington, USA, in 1994. The samples were frozen at -70°C until use.

MF-FAT. The MF-FAT procedure of Elliott & Barila (1987), as modified by Pascho et al. (1991), was used for these experiments. To disperse and digest cellular debris, a 0.5 ml coelomic fluid sample was mixed with 0.5 ml each of the following solutions: (1) phosphate-buffered saline containing 100 mg l^{-1} thimerosal as a preservative (PBS, 0.01 M phosphate, pH 7.1) and with 0.5% (by volume) Triton X-100 added (PBS-Triton), and (2) a trypsin solution prepared by mixing trypsin powder (Difco 1:250, Difco Laboratories, Detroit, MI, USA) at 10 g l^{-1} in distilled water at 4°C, then clarifying the mixture by filtration through Whatman No. 1 filter paper (Whatman, Maidstone, England) followed by filtration through a 0.2 µm filter. The mixture of coelomic fluid, PBS-Triton, and trypsin solution was heated at 50°C for 10 min, then filtered through a 13 mm diameter, 0.2 µm pore size polycarbonate filter (Nuclepore Corp., Cambridge, MA, USA) backed by a 5.0 µm nylon support filter (Micron Separations, Inc., Westboro, MA, USA) in a syringe-mounted disposable pop-top holder (Nuclepore). Filters were then rinsed with 3 ml of PBS-Triton delivered by syringe. For immunofluorescence staining, 100 µl of fluorescein isothiocyanate-(FITC) conjugated, affinity-purified immunoglobulin to *Renibacterium salmoninarum* (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA) was placed on each filter for 1 h at room temperature. The conjugate was used at a working dilution of 1:40 (v/v) in PBS and was filtered through a 0.2 µm filter before use. Each filter was rinsed as previously described and counterstained with 1 ml of Eriochrome black T (Sigma, St. Louis, MO, USA) prepared at 500 mg l^{-1} in PBS and filtered through Whatman No. 1 and Whatman No. 42 filter papers before initial use. Filters were removed from the holders, placed on glass microscope slides and air dried; cover glasses were mounted with glycerol-DABCO mounting medium (Johnson et al. 1982).

Positive control filters were prepared with coelomic fluid samples that had tested positive for *Renibacterium salmoninarum* by the ELISA and by the MF-FAT using both monoclonal antibodies and polyclonal antiserum. The control samples originated from spawning populations of spring chinook salmon that also had tested positive for *R. salmoninarum* by bacteriological culture. Because of a lack of known *R. salmoninarum*-negative populations of spring chinook salmon, negative control filters were prepared using reagents only, with sample diluent substituted for the coelomic fluid sample. These controls were used to ensure that reagents and filters did not become contaminated during MF-FAT procedures.

Each filter was examined by epifluorescence microscopy at 1000× magnification. A Zeiss WL microscope with a Neofluar 100× objective was used for all experiments except the second comparison of the MF-FAT

and S-FAT, for which a Zeiss Axiophot microscope with a Zeiss Planapochromat 100× objective was used. Both microscopes were equipped with filter sets for FITC. Bacteria showing specific fluorescence and having morphological characteristics consistent with *Renibacterium salmoninarum* were counted in 50 and 100 microscope fields on each filter. These counts were converted to cells per milliliter of the original coelomic fluid sample according to the formula of Ecker & Lockhart (1959) as adapted by Elliott & Barila (1987): cells ml^{-1} = (conversion factor) × (dilution factor) × (no. cells counted)/no. fields counted, where the conversion factor is the filtering surface area divided by the area of a single field at the magnification used.

S-FAT. Unless otherwise specified, coelomic fluid samples were centrifuged in microcentrifuge tubes (Sarstedt, Inc., Newton, NC, USA) for 10 min at 8800 × *g* in an Eppendorf 5413 centrifuge (Brinkmann Instruments, Inc., Westbury, NY, USA) equipped with a horizontal rotor. The supernatant was discarded, then the pellet was removed with a calcium alginate swab (Calgiswab Type 4, Spectrum Laboratories, Los Angeles, CA, USA) moistened with PBS (0.01 M, pH 7.1). The pelleted material was smeared thinly on both wells of a 2-well microscope slide (Cel-Line Associates, Newfield, NJ, USA; 8 mm diameter wells). The smears were air dried and then heat fixed by passing them over a flame. The slides were stained with the same preparation and working dilution of FITC-conjugated anti-*Renibacterium salmoninarum* immunoglobulin as used for the MF-FAT. A 50 µl volume of the conjugate was placed on each well of a slide, then the slide was incubated in a humid chamber at room temperature for 1 h. To minimize sloughing of the smears, slides were rinsed carefully with PBS, then flooded with PBS for 10 min. The slides were counterstained by flooding them for 60 s with an aqueous suspension (1:60 w/v) of Eriochrome black T, then the slides were rinsed as previously described. After a final rinse with distilled water, the slides were air dried and cover glasses were mounted with glycerol-DABCO medium. Positive control slides were prepared from the same known positive coelomic fluid samples as were used for the MF-FAT. Each slide was examined by epifluorescence microscopy, and fluorescing bacteria that met the criteria described for the MF-FAT were counted in 50 microscope fields (1000 × magnification) per well, for a total of 100 fields per slide. The counts were converted to cells per milliliter of the original coelomic fluid sample according to the formula of Ecker & Lockhart (1959) as previously described, but the total surface area of the slide wells was substituted for the total filtering surface area in the formula.

Comparison of MF-FAT and S-FAT. For the first experiment, coelomic fluid samples from 27 individual chinook salmon spawned at Dworshak NFH were used. These samples had been tested 3 yr previously by the MF-FAT, and had shown a range of *Renibacterium salmoninarum* concentrations from no detected bacteria to about 1.5×10^9 cells ml^{-1} when 150 microscope fields per filter were examined. Each sample was tested by the S-FAT and retested by the MF-FAT; a 0.5 ml volume of coelomic fluid was used for each test. The samples were coded so that they could be examined without bias.

The second experiment used coelomic fluid samples from 151 individual Carson NFH chinook salmon that had not been tested previously by either the MF-FAT or the S-FAT. Twenty-nine of the samples were taken from fish that had grossly visible kidney lesions characteristic of BKD; the remaining samples were from asymptomatic fish. Subsamples of 0.5 ml each were examined by each test. Based on the results of the first experiment, the S-FAT procedure was modified to further enhance retention of the smears on the slides. After heat-fixing a smear, it was dipped in acetone for 1 min, and allowed to drain. The PBS rinses were reduced from 10 min to 5 min.

Effect of centrifugation speed on S-FAT counts. A coelomic fluid sample was pooled from 10 female chinook salmon spawned at Dworshak NFH. By MF-FAT testing, the *Renibacterium salmoninarum* concentration in this sample was 5.4×10^5 cells ml^{-1}. Samples were centrifuged at 2000, 4000, 6000, 8000, and 10000 × *g* for 10 min in a Sorvall RC-5B centrifuge (Du Pont Co., Wilmington, DE, USA) equipped with a Sorvall SH-MT horizontal rotor. Four 0.5-ml subsamples were centrifuged at each speed. Bacteria were counted in 100 microscope fields per slide as previously described.

Statistical analysis. The Fisher exact test (Zar 1974) was used to compare the relative prevalences of *Renibacterium salmoninarum* detected in coelomic fluid samples by the MF-FAT and the S-FAT, and to compare the relative prevalences of the bacterium detected by a given FAT procedure when 50 and 100 microscope fields were examined on a slide or filter.

RESULTS

Comparison of MF-FAT and S-FAT

For the first experiment, examination of 100 rather than 50 microscope fields per sample did not increase the number of coelomic fluid samples positive for *Renibacterium salmoninarum* by the MF-FAT, and did not result in a significant increase ($p = 0.57$) in positive samples by the S-FAT (Table 1). However, the number of samples positive for *R. salmoninarum* was higher ($p <$

Table 1. Comparison of the MF-FAT and S-FAT for detection and quantification of *Renibacterium salmoninarum* in coelomic fluid of chinook salmon *Oncorhynchus tshawytscha* from Dworshak National Fish Hatchery, Idaho, USA. The 27 samples were selected because they had shown a wide range of *R. salmoninarum* concentrations (or were negative) by previous MF-FAT testing. The calculated detection limit for the MF-FAT in this experiment was 128 bacteria ml^{-1} when 50 fields were examined and 64 bacteria ml^{-1} when 100 fields were examined. The calculated detection limit for the S-FAT was 160 bacteria ml^{-1} when 50 fields were examined and 80 bacteria ml^{-1} when 100 fields were examined. TNTC: bacteria too numerous to count (see footnote). Significant difference at $^{*}p < 0.0001$ and $^{**}p < 0.0001$

Female no.	No. of *R. salmoninarum* counted			
	50 fields		100 fields	
	MF-FAT	S-FAT	MF-FAT	S-FAT
82	0	0	0	0
742	0	0	0	0
822	0	0	0	0
1208	1	0	1	0
1216	1	0	1	0
850	1	0	2	0
1158	1	0	2	0
786	1	0	3	0
854	1	0	3	0
998	1	0	3	0
800	3	0	4	0
1100	4	0	4	0
746	2	0	5	0
852	3	0	7	0
928	5	0	11	0
1058	9	0	12	0
834	11	0	17	0
952	15	0	38	1
798	33	0	72	0
964	40	1	86	1
762	80	0	154	1
32	358	17	714	27
1196	316	4	721	11
112, 122, 704, 172[a]	TNTC	TNTC	TNTC	TNTC
Total no. positive (%)	24(89%)*	7(26%)*	24(89%)**	9(33%)**

[a]Previous MF-FAT counts for these samples were: 112 = 8.9×10^6 cells ml^{-1}, 122 = 4.7×10^7 cells ml^{-1}, 704 = 7.0×10^8 cells ml^{-1}, and 172 = 1.5×10^9 cells ml^{-1}

0.0001) by the MF-FAT than by the S-FAT regardless of the number of microscope fields examined per filter or slide (Table 1). Seventeen more samples (a 63% difference in prevalence) were positive by the MF-FAT than by the S-FAT when 50 microscope fields were examined, and 15 more samples (a 56% difference in prevalence) were positive by the MF-FAT than by the S-FAT when 100 microscope fields were examined. *Renibacterium salmoninarum* were detected by the S-FAT only in samples for which the MF-FAT counts were ≥40 bacteria in 50 fields or ≥38 bacteria in 100 fields, representing calculated concentrations (by the MF-FAT) of 5.1×10^3 and 2.4×10^3 cells ml^{-1}, respectively. For samples in which *R. salmoninarum* were countable by both the MF-FAT and the S-FAT (Table 1), the counts of bacteria on the filters averaged 47 times higher (± 30 SD) than counts on the corresponding slides when 50 microscope fields were examined (3 samples), and averaged 74 times higher (± 51 SD) when 100 microscope fields were examined (5 samples).

Because of changes made in the MF-FAT procedures, this experiment was not intended as a direct comparison between the present MF-FAT analysis and that done on the same coelomic fluid samples 3 yr earlier. Nevertheless, the prevalence of *Renibacterium salmoninarum*-positive samples detected by the present MF-FAT after prolonged sample storage at −70°C (with additional thawing and freezing of some samples between analyses) was not significantly different ($p = 0.48$) from the prevalence (82%) detected by the earlier MF-FAT. One sample that tested positive by the earlier procedure was negative by the present analysis, and 3 samples that tested negative by the earlier procedure were positive by the present analysis. These differences were observed in samples with <10 bacteria counted in 100 microscope fields (present test) or 150 microscope fields (previous test); all samples with higher bacterial counts were positive in both studies. Among samples testing positive by both MF-FAT analyses, correlation of *R. salmoninarum* counts was high ($r^2 = 0.957$).

In the second experiment, examination of 100 microscope fields rather than 50 fields for each sample resulted in small increases in the numbers of coelomic fluid samples positive for *Renibacterium salmoninarum* by both FATs (Table 2), but the increases were not significant for either the MF-FAT ($p = 0.57$) or the S-FAT ($p = 0.90$). However, comparison of the MF-FAT to the S-FAT showed significantly more samples positive by the MF-FAT than by the S-FAT when either 50 microscope fields ($p = 0.0002$) or 100 microscope fields ($p < 0.0001$) were examined (Table 2). The MF-FAT detected 32 more positive samples than the S-FAT (a 21% difference in prevalence) when 50 microscope fields were examined, and 35 more positive samples than the S-FAT (a 24% difference in prevalence) when 100 microscope fields were examined. All samples that were positive for *R. salmoninarum* by the S-FAT also were positive by the MF-FAT. Single *R. salmoninarum* were detected by the S-FAT in samples showing as few as 3 bacteria in 100 fields and 5 bacteria in 50 fields by the MF-FAT. However, the bacterium was detected consistently by the S-FAT only in samples for which counts by the MF-FAT were ≥262 cells in 100 fields and ≥420 cells in 50 fields, representing calculated concentrations (by the MF-FAT) of 9.8×10^3 cells ml^{-1}

and 3.1×10^4 cells ml^{-1}, respectively. In 13 individual samples for which bacterial counts were obtained by both the MF-FAT and the S-FAT, the counts averaged 175 times higher (±165 SD) by the MF-FAT compared with the S-FAT when 50 microscope fields were examined and 174 times higher (±139 SD) by the MF-FAT when 100 microscope fields were examined.

Among the 29 coelomic fluid samples taken from fish with visible kidney lesions characteristic of BKD, 28 (97%) were positive for *Renibacterium salmoninarum* by the MF-FAT and 25 (86%) were positive by the S-FAT. More than 50 bacteria were observed in 50 or 100 microscope fields in samples from 26 of the lesioned fish positive by the MF-FAT and in samples from 23 of the lesioned fish positive by the S-FAT.

Effect of centrifugation speed on S-FAT counts

The number of bacteria detected by the S-FAT increased as the centrifugation speed increased (Fig. 1). Mean bacterial counts were more than 4 times greater for subsamples centrifuged at 10000 × *g* for 10 min than in samples centrifuged at 2000 × *g* or 4000 × *g* for 10 min. The coefficient of variation (CV) for the mean bacterial counts at a given centrifugation speed ranged from 28% at 8000 × *g* to 64% at 6000 × *g*. The highest calculated *Renibacterium salmoninarum* concentration obtained by S-FAT testing (8.7×10^3 cells ml^{-1}) was more than 6-fold lower than that obtained for the same sample by MF-FAT testing.

Table 2. Comparison of the MF-FAT and the S-FAT for detection and enumeration of *Renibacterium salmoninarum* in 151 coelomic fluid samples from chinook salmon *Oncorhynchus tshawytscha* at Carson National Fish Hatchery, Washington, USA. The samples had not been tested previously by either FAT The calculated detection limit for the MF-FAT in this experiment was 75 bacteria ml^{-1} when 50 fields were examined and 37 bacteria ml^{-1} when 100 fields were examined. The calculated detection limit for the S-FAT was 105 bacteria ml^{-1} when 50 fields were examined and 53 bacteria ml^{-1} when 100 fields were examined. Significant difference at $^{*}p = 0.0002$ and $^{**}p < 0.0001$

Total no. cells detected	No. samples in category (%) 50 fields		100 fields	
	MF-FAT	S-FAT	MF-FAT	S-FAT
0	78 (52%)	110 (73%)	73 (48%)	108 (72%)
1–10	27 (18%)	10 (7%)	27 (18%)	10 (7%)
11–25	2 (1%)	2 (1%)	5 (3%)	4 (2%)
26–50	3 (2%)	0	1 (1%)	0
>50	41 (27%)	29 (19%)	45 (30%)	29 (19%)
Total no. positive (%)	73 (48%)*	41 (27%)*	78 (52%)**	43 (28%)**

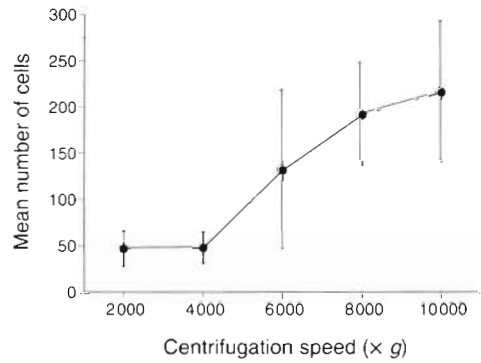

Fig. 1. Mean number (±SD) of *Renibacterium salmoninarum* cells counted in 100 microscope fields (1000× magnification) from coelomic fluid samples centrifuged at selected speeds for 10 min and examined by the S-FAT. Coelomic fluid pooled from 10 female chinook salmon *Oncorhynchus tshawytscha* naturally infected with *R. salmoninarum* was used; 4 subsamples were centrifuged at each speed

DISCUSSION

Renibacterium salmoninarum detection rates were 1.8 to 3.4 times higher by the MF-FAT than by the S-FAT in our study. These findings were similar to those of Lee & Gordon (1987b), who reported a 3-fold increase in the rate of detection of *R. salmoninarum* in coelomic fluid samples by an indirect MF-FAT in comparison to the S-FAT. Because Lee & Gordon (1987b) did not describe procedures such as centrifugation speed and time used for their S-FAT, direct comparisons with our S-FAT results are difficult.

In the present comparison, both FATs detected *Renibacterium salmoninarum* in the coelomic fluid from most fish showing grossly visible kidney lesions characteristic of BKD. The single lesioned fish that was negative by the MF-FAT also tested negative (both kidney and coelomic fluid) by an ELISA, suggesting that the lesion had a cause other than BKD.

Several factors may have contributed to the lower sensitivity of the S-FAT compared to the MF-FAT. The results of the centrifugation experiment suggested that not all of the bacteria were pelleted by centrifugation for 10 min at speeds up to 10000 × *g*, and that higher speeds or longer centrifugation times would be required for more complete pelleting of the bacteria. Bacteria may also have been retained on the swabs or may have become detached from the slides during the staining or rinsing steps. Because all steps of the MF-FAT are completed while the filters are retained in syringe-mounted holders, losses of bacteria are minimized (Elliott & Barila 1987).

Increasing the area examined on a filter or slide from 50 microscope fields to 100 microscope fields did not greatly increase the *Renibacterium salmoninarum*

detection rate by either the S-FAT or MF-FAT. The change in the number of fields examined resulted in a maximum difference in the detected *R. salmoninarum* prevalence of only 4% by the MF-FAT and 7% by the S-FAT.

The variability in S-FAT results, as evidenced in our study by the high CVs in the centrifugation experiment and inconsistent detection of *Renibacterium salmoninarum* by the S-FAT when MF-FAT counts were as high as 215 cells in 100 microscope fields, was similar to that reported by other authors. Armstrong et al. (1989) described the testing of coelomic fluid samples from 184 chinook salmon by 2 independent laboratories using an S-FAT in which samples were centrifuged at 10000 × *g* for 10 min. The pellets were resuspended in the small amount of remaining coelomic fluid, then thin smears were made on slides and subsequently stained by an indirect FAT. When the observation of one or more cells in 50 microscope fields at 1000 × magnification was considered a positive result, agreement between the results of the 2 laboratories could be explained entirely by chance. When only those samples with more than 50 cells observed in 50 fields were regarded as positive, agreement between the results of the 2 laboratories was considered statistically significant, but the level of agreement was deemed unsatisfactory for a population screening test. From comparisons with bacteriological culture, Armstrong et al. (1989) estimated that about 60% of the S-FAT results in their study were falsely negative.

Although our experiments did not compare the variability of repeated MF-FAT counts with that of repeated S-FAT counts, the results of previous studies indicate that the variability of MF-FAT counts is lower. Elliott & Barila (1987) filtered 10 subsamples of a coelomic fluid sample pooled from *Renibacterium salmoninarum*-positive fish (similar to the sample used for our S-FAT centrifugation test), and reported a CV of about 7% when 100 microscope fields were examined per filter by the MF-FAT. Lee & Gordon (1987b) prepared 3 filters from a broth culture of *R. salmoninarum*, and observed a CV of about 5% for counts of 100 microscope fields per filter by an indirect MF-FAT. Both Elliott & Barila (1987) and Lee & Gordon (1987b) reported increased CVs when fewer fields were examined per filter, or after 2-fold serial dilutions of samples were made before filters were prepared. Nevertheless, the CVs for MF-FAT counts in those studies remained below 20% even when samples were diluted 1:16 (v/v) before filtration and only 50 fields per filter were examined, whereas 28% was the lowest CV observed in the present study for S-FAT counts of 100 microscope fields per slide on 4 slides. Elliott & Barila (1987) and Lee & Gordon (1987b) also observed good correspondence between MF-FAT counts and plate counts of *R. salmoninarum* broth cultures, with slightly higher numbers of bacteria detected by the MF-FAT.

Even though it is more sensitive than the S-FAT, the present MF-FAT procedure can be more labour-intensive and time-consuming for the testing of large numbers of samples. Modifications should be considered, including the use of inorganic filters that would allow more rapid filtration of samples (Jones et al. 1989), or the use of manifolds that would enable the simultaneous filtration of multiple samples (S. Foott, U.S. Fish and Wildlife Service, California, pers. comm.).

The MF-FAT and other techniques are now being used to investigate the relation between *Renibacterium salmoninarum* concentrations in coelomic fluid and vertical transmission of the bacterium. Evidence suggests that the probability of vertical transmission increases with increasing concentrations of *R. salmoninarum* in the coelomic fluid (Lee & Evelyn 1989, Pascho et al. 1991). Nevertheless, transmission of the organism from fish with extremely low infection levels may occur. The bacterium has been detected by the polymerase chain reaction (PCR) in eggs from female chinook salmon that had tested negative for *R. salmoninarum* infection by both the ELISA and the MF-FAT (Brown et al. 1994).

The greater sensitivity of the MF-FAT compared to the S-FAT indicates that the MF-FAT would be preferable for use in situations such as inspections requiring the detection of low numbers of bacteria. Cross-reactions of other bacterial species with antisera prepared against *Renibacterium salmoninarum* have been reported (Bullock et al. 1980, Austin et al. 1985, Yoshimizu et al. 1987, Brown et al. 1995), so the inclusion in any FAT of control material from known *R. salmoninarum*-positive fish is necessary for comparison of cell morphology and staining properties of bacteria in test and control samples. Because neither the MF-FAT or the S-FAT can distinguish live from dead *R. salmoninarum*, culture of important samples may be advisable for confirmation of viability.

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