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

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A simple, rapid PCR assay for the identification of Renibacterium salmoninarum in Atlantic salmon (Salmo salar L.) tissues detected DNA extracted from between 4 and 40 bacterial cells. PCR was at least as sensitive as culture when it was used to identify subclinically infected fish experimentally challenged with R. salmoninarum. However, PCR identified much higher numbers of kidney tissue and ovarian fluid samples from commercially reared broodstock fish to be positive for R. salmoninarum than did culture. This difference may be due to the antibiotic chemotherapy of broodstock fish used by the industry in 1994 to control the vertical transmission of R. salmoninarum. A much closer relationship between PCR and culture results was observed for ovarian fluid samples collected from broodstock fish in 1993. Also, PCR scored a much higher percentage of kidney tissue samples than ovarian fluid samples from 1994 broodstock fish positive for R. salmoninarum, which may reflect the uneven distribution of the pathogen in different fish tissues. Inclusion of a nested probe to identify the PCR-positive 1994 ovarian fluid samples increased the sensitivity of detection to between one and four cells and the number of samples that scored positive by almost threefold. These data indicate that many infected ovarian fluid samples contained very low numbers of R. salmoninarum cells and, because almost all these samples were culture negative, that PCR may have detected dead or otherwise unculturable bacterial cells.

Bacterial kidney disease (BKD) caused by Renibacterium salmoninarum is one of the most important diseases affecting cultivated salmonids worldwide (for a review, see reference 9). The disease can be transmitted horizontally to cohabiting salmonids and vertically through infected eggs to progeny. It is commonly a chronic, granulomatous, and often fatal infection, although acute infections may occur. Control of the disease in cultivated salmonids is hampered by the occurrence of subclinically infected carrier fish, the lack of effective chemotherapy, and the absence of an efficacious vaccine.

Culling of subclinically infected broodstock fish is considered to be the most effective means of controlling transmission of the pathogen. This is accomplished by screening ovarian fluid surrounding the eggs, in addition to kidney tissue, for the presence of R. salmoninarum. Commonly used techniques for this purpose are the indirect fluorescent-antibody assay (7) and the enzyme-linked immunosorbent assay (13, 21). However, both methods lack the required sensitivity to detect many of the subclinically infected fish (2, 11). While cultivation on selective kidney disease medium (SKDM) (3) is the more relevant and sensitive method for the detection of viable R. salmoninarum, the long incubation times (6 or more weeks at 15°C) (4) required before individual colonies can be observed make it much less practical.

This has led to recent interest in PCR assays for the detection of R. salmoninarum from infected fish (18) as well as individual salmonid eggs (5). In addition, a reverse transcriptase PCR assay has been used to detect R. salmoninarum rRNA extracted from ovarian fluids (20). Screening of a significant number of individual eggs from each female broodstock fish may prove to be impractical, and it may be necessary to simplify such assays before they are accepted for use for routine diagnosis.

In the study described in this report, a simplified PCR assay was investigated for its sensitivity and practicality at detecting R. salmoninarum in kidney tissue and ovarian fluids from Atlantic salmon collected during spawning activities in New Brunswick, Canada. The PCR assay was compared to culture for the detection of subclinically infected, experimentally challenged fish. The PCR assay and a nested probe PCR assay were subsequently evaluated and compared to culture for the routine screening of commercially reared broodstock fish.

MATERIALS AND METHODS

Bacterial strains. The two R. salmoninarum strains used throughout this study were isolated from Atlantic salmon (Salmo salar L.) during outbreaks of BKD at salmon farms on the Bay of Fundy (strain F91) and Miramichi River (strain KDP-2) in New Brunswick, Canada (19), and were subcultured on SKDM agar (3) for 3 weeks at 15°C. Confluent lawns of bacteria were washed off the agar plates with sterile distilled deionized H2O (ddH2O). Aliquots of 50 μl containing approximately 1.5 × 108 cells were stored at −70°C until used. Cell numbers were estimated by using the absorbance of the cell suspensions (an optical density at 660 nm of 1.0 corresponded to approximately 107 cells ml−1) from hemocytometer counts of cell suspensions dispersed in 0.05% [vol/vol] Triton X-100. R. salmoninarum autoagglutinates (6) and drop-plate cultures (12) of the bacterial strains used throughout this study were subcultured at 37°C (Staphylococcus aureus ATCC 12600, Staphylococcus epidermidis ATCC 155, Escherichia coli ATCC 11303, and Pseudomonas aeruginosa ATCC 10145) or 26°C (Micrococcus luteus ATCC 4698, Bacillus subtilis ATCC 2789, Serratia marcescens ATCC 21743, and Aerobacter aerogenes ATCC 13048) on Trypticase soy agar. Aliquots of these bacterial strains were harvested and stored as described above. For
convenience, a crude estimate of 10^9 cells ml^-1 at an optical density of 1.0 at 660 nm was used for these strains because they were tested at concentrations well in excess of the R. salmoninarum cell concentrations. Hemocytometer cell counts were determined only for the E. coli and A. salmonicida strains, which corresponded to 0.5 to 1.0 x 10^9 cells ml^-1 at an optical density of 660 nm of 1.0. Serial dilutions of the harvested bacterial strains in ddH2O were used to establish the specificities of the PCR assays.

Extraction of DNA from bacterial stains for PCR. Aliquots (20 μl) of harvested bacterial suspensions were centrifuged at 13,000 x g for 1 min in a microcentrifuge, and the cell pellets were washed twice with 1 ml of ddH2O. Instagene matrix (200 μl; Bio-Rad Laboratories Ltd., Hercules, Calif.) was added to the cell pellets, and the mixture was incubated at 55°C for 30 min, vortexed for 10 s, and pelleted twice in a boiling water bath for 8 min. The samples were vortexed, centrifuged, and the supernatant was subjected to PCR or was stored at −20°C until assayed. For R. salmoninarum cell suspensions, the extracted DNA concentration was estimated by measuring the absorbance at 260 nm with a Gene Quant RNA/DNA Calculator (Pharmacia Biotech Inc., Uppsala, Sweden), and the corresponding cell number was derived by using the estimated value of 4 of DNA per R. salmoninarum cell (18). The cell numbers generated from the extracted DNA concentration were similar (within approximately onefold) to the cell numbers estimated from the absorbance of the cell suspensions.

DNA primers for PCR-based assays for R. salmoninarum. DNA primer pairs were selected from the published gene sequence of the major p57 protein antigen (8) produced by R. salmoninarum isolates from a wide geographic range (10). The primers were designed with the assistance of the Oligo Software Program (National Biosciences Inc., Plymouth, Minn.) and were synthesized by using a Cyclone Plus DNA Synthesizer (Milligan/Biotech, Navato, Calif.) according to the manufacturer’s instructions. The nucleotide sequences of the primers were Forward (positions 249 to 269) (FL7) (5'-AGCAGGAGGACCAGTTGCAG-3'), Reverse (positions 598 to 578) (RL11) (5'-GGTGTAACGATAAATGGCCCTCCGTA-3'), Forward (positions 621 to 611) (RL5) (5'-TTCCGTTCCTCGGTTATCCTGCTCT-3'), and Forward (positions 449 to 469) (FL10) (5'-GGGTTAACGATAAATGGCCCTCCGTA-3'). The primer pair FL7-RL11 was chosen to amplify a 149-bp segment (nucleotides 249 to 598 inclusive), the pair FL7-RL5 was chosen to amplify a 349-bp segment (nucleotides 249 to 621 inclusive), and the pair FL10-RL11 was chosen to amplify a 149-bp segment (nucleotides 449 to 598 inclusive).

PCR assays for R. salmoninarum. Amplification was performed in 50 μl containing KCl, 50 mM; Tris-HCl (pH 8.3), 10 mM; MgCl₂, 1 mM; each nucleotide triphosphate, 0.1 mM; each primer, 0.17 μM; Taq polymerase (Gibco BRL Life Technology Inc., Grand Island, N.Y.), 1.5 U; Taq start antibody (Clonetch Laboratories Inc., Palo Alto, Calif.), 1.5 μl of antibody dilution; and various concentrations of DNA as the template (in a 10-μl volumes). The reaction mixture was centrifuged at 13,000 x g for 20 s, overlaid with 70 μl of mineral oil, centrifuged again, and placed in a PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, Mass.). For primer pairs FL7-RL11 and FL10-RL11, the incubation conditions were 94°C for 5 min, followed by 35 cycles of 93°C for 1 min (denaturing), 60°C for 1 min (annealing), and 72°C for 30 s (extending) and a final incubation at 72°C for 2 min. For primer pair FL7-RL5, the incubation conditions were 94°C for 2 min, followed by 5 cycles of 94°C for 15 s (denaturing), 63°C for 2 min (annealing), and 72°C for 15 s (extending) and 35 cycles of 94°C for 15 s, 63°C for 15 s, and 72°C for 15 s and a final incubation at 72°C for 1 min. The reaction was carried out in a Gene Amp 9600 instrument (Perkin-Elmer, Foster City, Calif.). These conditions were determined by varying the MgCl₂, nucleotide triphosphate, and primer concentrations and the annealing and extension temperatures and times to optimize the amplification of the desired products and to minimize the appearance of secondary products. The amplified DNA products (10-μl volumes) were subjected to electrophoresis on polyacrylamide gels (percent as indicated below) at 200 V of constant voltage by using a Mini Protein II electrophoresis apparatus (Bio-Rad Laboratories Inc.) and visualized by silver staining (silver stain kit; Bio-Rad Laboratories Inc.). To verify correct amplification with primer pairs FL7-RL11 (349 bp) and FL7-RL5 (372 bp), amplified material was separated by electrophoresis in 1.8% low-merging-temperature agarose gels and stained with ethidium bromide, and the PCR product band was excised. The agarose containing the PCR products was melted at 70°C, and the resulting solution processed with Wizard DNA Clean Up columns (Promega Corp., Madison, Wis.) to isolate the DNA according to the manufacturer’s instructions. The recovered 349- and 372-bp products were then used as the template for PCR with the internal primer pair FL10-RL11, and the amplified product was subjected to polyacrylamide gel electrophoresis and visualized as described above.

Probe-PCR assay for R. salmoninarum. Primers FL10 and RL11 were used to amplify the 149-bp segment of the p57 gene from DNA extracted from cultured R. salmoninarum. The 149-bp segment was isolated as described previously (8). The probe was labelled with psoralen biotin by using the Rad-Free kit (Schleicher & Schuell, Inc., Keene, N.H.) according to the manufacturer’s instructions. PCR was performed with samples by using primers FL7 and RL5, and the PCR product was subjected to polyacrylamide gel electrophoresis (as described above), transferred to nitrocellulose membranes, and hybridized with the labelled 149-bp internal probe according to the manufacturer’s instructions (Schleicher & Schuell, Inc.), except that the chemiluminescent alkaline phosphatase substrate was CSPD (disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2-(5′-chloro)-tricyclo [3.3.1.1^9]heptane]-4′-yloxy) phosphatase) (Tropi/Porcian, Mississauga, Ontario, Canada). The amplified DNA products (10-μl volumes) were exposed to X-ray film for 1 h at 37°C to visualize the amplified product.

Collection and processing of kidney tissue and ovarian fluid samples from Atlantic salmon. Kidneys were removed from sampled Atlantic salmon and were homogenized (10% [wet weight]) as reported previously (12). For the initial test (see Fig. 2), samples (50 μl) of tissue homogenates were placed on glass fiber filters (no. 30; Schleicher & Schuell, Inc.) and were dried overnight at room temperature. For subsequent assays, 50-μl samples of kidney homogenates were added to 1 ml of ddH2O, centrifuged at 13,000 x g for 30 s, washed with 1 ml of ddH2O, and centrifuged again. The washed pellet was suspended in 50 μl of ddH2O and was placed on a glass fiber filter as described above. The DNA was extracted from the glass fiber filters by using the Instagene Matrix as described above.

Ovarian fluid samples were collected from gravid female fish as reported previously (11). For the initial test (see Fig. 2), 50-μl samples of the ovarian fluids were placed on glass fiber filters and treated as described above. For subsequent assays, ovarian fluid cellular debris was concentrated by centrifuging 3-ml samples at 2,000 x g for 10 min (model HN; International Equipment Co., Needham Heights, Mass.), and the pellet was extracted with Instagene Matrix as described above.

Sensitivity of PCR with seeded samples and experimentally challenged Atlantic salmon. Presumed negative (by PCR and culture on SKDM agar) stored (−20°C) kidney tissue homogenates and ovarian fluids were seeded with serial dilutions of cultivated R. salmoninarum, the DNA was extracted, and PCR was performed as described above.

To compare the sensitivity of PCR with that of culture of kidney tissue to identify R. salmoninarum in infected fish, Atlantic salmon (weight, 15 to 30 g) (each) were acclimated and maintained at 10 to 11°C as described previously (19) and were then challenged with the pathogen. A total of 36 fish were injected intraperitoneally with approximately 2 x 10^7 CFU of R. salmoninarum in 0.2 ml of phosphate-buffered saline, the fins were clipped for identification, and the fish were placed in a tank containing 61 unchallenged fish to initiate a low-level BDK infection in the cohort unchallenged fish. Any fish that died were removed twice daily and were checked for internal clinical signs of BDK (9) and for R. salmoninarum by culture of kidney tissue on SKDM agar. At week 9 postchallenge, the remaining fish were sacrificed and were checked for R. salmoninarum by both culture of kidney tissue on SKDM agar and PCR of kidney tissue. The numbers of R. salmoninarum CFU per gram (wet weight) of kidney tissue were determined by drop plating 10-μl aliquots of serial dilutions of 10% kidney homogenates onto the agar plates and incubating the plates for 6 weeks at 15°C (12). Colonies were identified as R. salmoninarum by Western blotting (immunoblotting) as reported previously (12, 19). PCR was performed as described above.

Analysis of kidney tissue and ovarian fluid collected from commercially reared broodstock Atlantic salmon. Samples of kidney tissue and ovarian fluid were collected from broodstock Atlantic salmon from farms on the Bay of Fundy, New Brunswick, and were assayed by culture, PCR, and probe-PCR as described above.

RESULTS

PCR detection of R. salmoninarum. DNA fragments of 349 and 372 bp were amplified from DNA extracted from cultivated cells of R. salmoninarum F91 and KDP-2 by using primer pairs FL7-RL11 and FL7-RL5, respectively. These amplified products were consistently detected on silver-stained polyacrylamide gels by using DNA extracted by a simple single step (Instagene Matrix; Bio-Rad Laboratories, Inc.) from between 4 and 40 bacterial cells (Fig. 1A). DNA extracted from cultivated cells of a variety of other bacteria tested (see text) did not yield a positive result with the same primer pairs because no amplified DNA bands of the appropriate sizes were detected (Fig. 1B).

Use of the isolated 349- or 372-bp amplified products as templates for the internal primers FL10 and RL11 produced the expected 149-bp amplified DNA band and confirmed that the expected R. salmoninarum DNA sequences were being amplified (Fig. 1C).

Detection of R. salmoninarum DNA in seeded Atlantic salmon kidney tissue and ovarian fluid samples. DNA was extracted from presumed negative (by culture and PCR) Atlantic salmon unwashed kidney tissue homogenates (10% [wet weight]...
polyacrylamide gels (8% in panels A and C; 12.5% in panel B).

cells (C). Arrowheads indicate the position of the 349 bp DNA product on homogenates (10% [wet wt/vol]; 50 
tracted from Atlantic salmon (S. salar L.). Samples were amplified with primers FL7 and RL11, electrophoresed, and silver stained as described in the legend to Fig. 1. (A) Amplification reactions (50-µl volume) contained DNA extracted from unwashed, pelleted (2,000 x g for 10 min) material from 37.5 µl of ovarian fluid. Ovarian fluid samples positive (lanes 1 to 3) and negative (lanes 4 and 5) for the 349-bp DNA product were tested. (B) Amplification reactions (50-µl volume) contained DNA extracted from 2.5 µl of washed (two times with ddH2O) kidney homogenates (10% [wet wt/vol]). Kidney samples positive (lanes 2 to 6) and negative (lanes 7 and 8) for the 349-bp DNA product were tested. Lane 1, amplification products of DNA extracted from 4 × 102 R. salmoninarum F91 cells; lane 2, 1-kb DNA ladder; lane 3, 20 ng of a 1-kb DNA ladder; lane 4, A. salmonicida S-Rest; lane 5, M. luteus ATCC 4698 (4 × 104 cells); lane 6, R. salmoninarum F91 (40 cells). Extracted DNA (from the cell concentrations described above in a 10-µl volume) was amplified (50-µl reaction volume) with primers FL7 and RL11, and the samples (10 µl) were subjected to polyacrylamide (8%) gel electrophoresis and silver staining (Bio-Rad Laboratories Inc.). (C) Isolated 349-bp product of primers (FL7 and RL11) amplified with primers FL10 and RL11, electrophoresed, and stained as described above. Arrowheads indicate the positions of the 349- and 149-bp DNA products.

wt/vol) seeded with R. salmoninarum cells. The sensitivity of PCR detection of the R. salmoninarum DNA with these extracts (Fig. 2A) was lower compared to that with DNA extracted directly from cultured R. salmoninarum cells. The decreased sensitivity was dependent on the concentration of extracted kidney tissue homogenate used (Fig. 2B). At the kidney tissue extract concentrations tested, washing of the kidney tissue homogenate (twice with ddH2O) prior to DNA extraction increased PCR sensitivity to the level previously observed with DNA extracts from cultured R. salmoninarum cells and greatly improved the detection of naturally infected kidney homogenate samples from broodstock Atlantic salmon (compare Fig. 3B and C). No similar reduction in PCR sensitivity was observed with extracts of unwashed, presumed negative ovarian fluid samples seeded with R. salmoninarum cells at the extract concentrations tested (Fig. 2C).

Some DNA bands (e.g., at approximately 1,200 bp) other than the intended target DNA were often seen on silver-stained gels of amplified samples containing R. salmoninarum DNA (Fig. 1 and 2). These additional bands may be due to a mismatch of the primer outside the intended target DNA. The gene sequence of the p57 protein is characterized by several repeat regions (8), which may result in some annealing of the primer outside the target region but within the p57 gene sequence, resulting in “jumping PCR” (16). However, the additional bands were not a hindrance to the interpretation of the results, because the intended 349- and 372-bp products were consistently detected in samples containing R. salmoninarum DNA.

Comparison of PCR and culture for the detection of R. salmoninarum in experimentally challenged Atlantic salmon. A low-level BKD infection was established by cohabitation of previously unchallenged fish with fish injected intraperitoneally with R. salmoninarum. Fish challenged by cohabitation were sampled at week 9 postchallenge, by which time most of the intraperitoneally challenged fish (28 of 36) had died. All fish that died displayed typical symptoms of BKD (e.g., white granulomatous lesions in kidney tissue) and the presence of R. salmoninarum following cultivation of kidney tissue on SKDM agar. Most of the live-sampled fish challenged by cohabitation were culture positive (21 of 27), with low numbers of R. salmoninarum CFU per gram (wet weight) of kidney tissue (Table 1) and no overt clinical signs of BKD. Washed kidney homog-
enates from all of the culture-positive fish and two of the six culture-negative fish scored positive by PCR, suggesting that PCR was at least as sensitive if not more sensitive than culture in identifying the subclinically infected fish.

**Comparison of PCR and culture for detection of R. salmoninarum in commercially raised broodstock Atlantic salmon.**

Of 151 ovarian fluid samples collected from fish on a farm in the Bay of Fundy in 1994, 23 (15.2%) scored positive for *R. salmoninarum* by PCR (Fig. 3A; Table 2). Unexpectedly, only 1 (<1%) sample was identified to be positive by culture. Higher percentages of culture-positive ovarian fluid samples had been identified from those collected from this farm in both 1992 (12.1%) and 1993 (12.2%). However, antibiotic chemotherapy of the broodstock fish prior to spawning (and sampling) was introduced by the industry in 1994 in an attempt to control the vertical transmission of BKD. Therefore, 50 stored (−20°C) ovarian fluid samples available from those samples collected in 1993 were assayed by PCR, and 5 scored positive, including all four of the samples previously identified to be positive by culture (Table 2).

A sample (*n* = 36) of broodstock fish was obtained from this farm in 1994, and analysis of washed kidney tissue homogenates showed that 18 samples were positive for *R. salmoninarum* by PCR (Fig. 3B). Similar to the ovarian fluid samples, only 1 kidney sample was identified to be positive by culture. The higher percentage of kidney samples (50%) positive by PCR compared to the percentage of ovarian fluid samples (15.2%) positive by PCR may suggest the presence of lower numbers of *R. salmoninarum* cells in ovarian fluid than in kidney tissue from subclinically infected broodstock fish.

**TABLE 1. Comparison of culture and PCR assay for the detection of *R. salmoninarum* in kidney tissue from live-sampled, experimentally challenged Atlantic salmon**

<table>
<thead>
<tr>
<th>Challenge method</th>
<th>CFU of <em>R. salmoninarum</em>/g (wet wt) of kidney tissue</th>
<th>No. of fish sampled</th>
<th>No. of sampled fish positive by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohabitation^a^</td>
<td>Not detected (1 × 10^0–10^2)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>≥1 × 10^3–&lt;1 × 10^5</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>&gt;1 × 10^5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intrauterine injection</td>
<td>&gt;1 × 10^6</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

^a After 6 weeks of incubation at 15°C on SKDM (3).

^b Fish were sampled at 9 weeks postchallenge.

^c Detection of amplified DNA product on silver-stained polyacrylamide gels.

**TABLE 2. Comparison of culture and PCR assay for the detection of *R. salmoninarum* in ovarian fluid samples from broodstock Atlantic salmon**

<table>
<thead>
<tr>
<th>Year collected</th>
<th>No. of samples</th>
<th>No. of samples positive for <em>R. salmoninarum</em></th>
<th>Culture^a^</th>
<th>PCR^a^</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>199</td>
<td>24</td>
<td>NP^b^</td>
<td>NP^b^</td>
</tr>
<tr>
<td>1993</td>
<td>89</td>
<td>13</td>
<td>NP^b^</td>
<td>NP^b^</td>
</tr>
<tr>
<td>1994</td>
<td>151</td>
<td>50^c^</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

^a See footnote a of Table 1.

^b See footnote b of Table 1.

^c Stored (at −20°C for 1 year) samples assayed by PCR.

To increase the sensitivity of PCR detection of *R. salmoninarum* in ovarian fluid, an internal chemiluminescence-labelled DNA probe (149-bp product amplified from *R. salmoninarum* DNA with primers FL10 and RL11) was used to detect the PCR products amplified from samples with primers FL7 and RL5. This assay increased the sensitivity of detection of *R. salmoninarum* to between one and four extracted cells (Fig. 4A), and by this assay the number of positive ovarian fluid samples obtained in 1994 increased to 65 of 151 (approximately 43%); among these positive samples were all 23 samples that scored positive by simple PCR (Fig. 4B). In order to detect *R. salmoninarum* in all nine samples, while simple PCR identified *R. salmoninarum* in eight of the samples (data not shown), the one culture-positive ovarian fluid sample not identified to be positive by simple PCR produced only two colonies on the SKDM agar plate.

**FIG. 4. Southern hybridization for the sensitivity of *R. salmoninarum* DNA detection (A) and analysis of representative ovarian fluid samples from commercially raised broodstock Atlantic salmon (*S. salar L.*) (B). (A) DNA was extracted from *R. salmoninarum* cells. Serial 10-fold dilutions were amplified (50-μl reaction volume) with primers FL7 and RL5, subjected to polyacrylamide (8%) gel electrophoresis (as described in the legend to Fig. 1), transferred to a nitrocellulose membrane, and hybridized to the internal 149-bp chemiluminescence-labeled probe (amplified from *R. salmoninarum* DNA with primers FL10 and RL11), and the membranes were developed on X-ray film. The amplification reactions contained DNA equivalent to 4 × 10^7^ (lane 1), 4 × 10^5^ (lane 2), 4 × 10^3^ (lane 3), 4 × 10^2^ (lane 4), 4 (lane 5), and 0.4 (lane 6) *R. salmoninarum* cells by using the estimated value of 4 fg of DNA per *R. salmoninarum* cell (18). Lane 7, H2O. (B) DNA was extracted from ovarian fluid samples (as described in the legend to Fig. 3) and treated as described above. Ovarian samples positive (as indicated by the arrowhead) (lanes 1, 3, 5, and 8) and negative (lanes 2, 4, 6, and 7) for the amplified product of primers FL7 and RL5 were tested.**

**DISCUSSION**

The initial objective of this study was to evaluate a simple PCR assay as a routine screening procedure to assist the aquaculture industry in controlling the vertical transmission of BKD in Atlantic salmon. The assay used could be performed easily within 24 h and detected *R. salmoninarum* DNA extracted from between 4 and 40 cells from either kidney tissue or ovarian fluid. Kidney tissue homogenates, at the concentrations used in the assay, required washing prior to DNA extraction, presumably because of the presence of inhibitory compounds (14, 20).

Analyses of kidney tissue homogenates from Atlantic salmon experimentally challenged with *R. salmoninarum* showed that the PCR assay was at least as sensitive if not more sensitive than the culture method used to identify subclinically infected fish.
ected fish. PCR identified all culture-positive samples, and culture identified most of the PCR-positive samples.

However, analysis of kidney tissue and ovarian fluid samples collected from commercially raised broodstock fish in 1994 demonstrated that culture identified very few of the PCR-positive samples. It is possible that the large discrepancy between PCR and culture for these samples may relate to the use by the industry in 1994 of antibiotic chemotherapy of the broodstock fish from which the samples were subsequently collected. The antibiotics may have killed many of the R. salmoninarum cells present in the tissues or otherwise rendered them nonculturable (22). There may be a period of time when target DNA sequences from nonviable bacterial cells are still available for PCR (15, 17). The number of culture-positive ovarian fluid samples collected from one farm in 1994 (<1% positive) was lower than might have been expected on the basis of the culture results obtained for the ovarian fluid samples collected from the same farm in 1992 (12.2% positive) and 1993 (12.1% positive), when antibiotic chemotherapy was not used. In fact, PCR analysis of stored ovarian fluid samples collected in 1993 showed a much closer relationship between PCR and culture results.

Also, a high number of PCR-positive kidney tissue samples (18 of 36) in a sample of broodstock fish from this farm in 1994 was observed compared to the number of PCR-positive ovarian fluid samples (23 of 151) collected from fish at the same site. The results may reflect the unequal distribution of R. salmoninarum cells between kidney tissue and ovarian fluid in subclinically infected female fish (which has been observed previously [11]) and raised the possibility that some of the ovarian fluid samples may have contained low, insufficient numbers of R. salmoninarum cells to be detected by simple PCR. This was shown to be the case because the probe-PCR assay, which increased the sensitivity of R. salmoninarum detection (DNA extracted from between one and four cells), increased the number of these ovarian fluid samples that scored positive for R. salmoninarum by almost threefold (65 of 151 samples). Unfortunately, the use of a probe-PCR assay adds complexity to the monitoring system. While it is not understood what levels of R. salmoninarum cells in ovarian fluid would indicate a significant hazard for the vertical transmission of the pathogen (intraovum infection), considering the historic problems associated with BKD in farmed salmon (9), it might be prudent to consider using such an assay.

An important question arising from the results presented here is whether the majority of PCR-positive ovarian fluid (and kidney) samples collected in 1994 and which were not positive by culture actually contained viable R. salmoninarum cells [i.e., there may be a window of opportunity for PCR to detect dead cells (15, 17)]. This distinction between viable and dead pathogenic cells may become important to the industry if antibiotic chemotherapy of broodstock fish prior to spawning is frequently used as a method to control the vertical transmission of R. salmoninarum. It may be possible to answer this question by developing a reverse transcriptase PCR assay to detect R. salmoninarum mRNA. The half-life of bacterial mRNA is comparatively short (1), and its detection would be more indicative of the presence of viable pathogen. This system is being developed.

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