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Real-time PCR for quantification of viable *Renibacterium salmoninarum* in chum salmon *Oncorhynchus keta*

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ABSTRACT: Quantification of *msa* gene mRNA of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), was investigated using reverse transcription followed by real-time PCR assay on *R. salmoninarum* in culture, and in experimentally challenged chum salmon *Oncorhynchus keta* fry kidney tissues (total of 70 samples) after intraperitoneal (i.p.) injection and bath infection. Correlations of *msa* gene mRNA concentrations with culturable cell concentrations (as colony forming units [CFU]), determined by drop-plate culture method on selective kidney disease medium (SKDM) agar through a 12 wk incubation time, and *msa* gene DNA concentrations by real-time PCR assay were examined. Furthermore, ovarian fluid samples from wild chum salmon adults with no clinical signs of disease were collected from 8 rivers and from clinically infected kokanee *O. nerka* and masu salmon *O. masou* that were reared in 1 and 2 hatcheries, respectively (total of 414 samples). All samples were examined by nested PCR assay. Then, positive samples were examined by real-time PCR assays for mRNA and DNA; mRNA was detectable at 8 log units (5.0×10^1 to 5.0×10^9 copies μl^{-1}) with high correlation ($R^2 = 0.999$). The mRNA concentration correlated with CFU in kidney tissue from fish infected by i.p. injection ($R^2 = 0.924$), by bath infection ($R^2 = 0.502$) and in culture ($R^2 = 0.888$). *R. salmoninarum* was detected and quantified by real-time PCR assay for mRNA in ovarian fluid samples in both subclinically infected chum salmon adults and clinically infected kokanee and masu salmon adults; detection rates ranged from 0 to 44.4 % and concentrations ranged from 9.7×10^2 to 5.6×10^5 copies μl^{-1}. These results indicate that real-time PCR assay for the mRNA is a rapid, sensitive and reliable method to detect and quantify the viability of *R. salmoninarum* in kidney and ovarian fluid samples of salmonid fishes with both clinical and subclinical infection of the pathogen.

KEY WORDS: Real-time PCR · *msa* gene mRNA · *Renibacterium salmoninarum* · Bacterial kidney disease · Viability · Chum salmon

INTRODUCTION

Bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* is one of the most important diseases affecting wild and cultured salmonid fishes worldwide (Fryer & Sanders 1981, Evenden et al. 1993). Among salmonid fish species cultivated in Japan, chum salmon *Oncorhynchus keta* is the most abundant; more than 2 billion fry are reared in hatcheries and released into rivers annually. The harvest reaches approximately 60 million adults in Japanese coastal waters. Chum salmon are susceptible to infection by *R. salmoninarum* experimentally, although the incidence of BKD has not been reported (Kimura et al. 1987, Sakai et al. 1991). Other salmonid fish species cultivated in Japan, including masu salmon *O. masou* and kokanee *O. nerka*, are also susceptible to infection by *R. salmoninarum*, and the disease has been reported in hatcheries (Sakai et al. 1986, 1991, Kimura et al. 1987).

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Control of the disease requires a rapid, sensitive and quantitative method for detection of culturable *Renibacteriium salmoninarum* or estimation of the viability of *R. salmoninarum* in clinically and subclinically infected fish. To screen for infected fish, the fluorescent antibody technique (FAT) (Evelyn et al. 1981, Armstrong et al. 1989, Cvitanich 1994), membrane filtration-fluorescent antibody technique (MF-FAT) (Elliott & Barila 1987), enzyme-linked immunosorbent assay (ELISA) (Turuga et al. 1987, Pascho & Mulcahy 1987, Pascho et al. 1991, Rockey et al. 1991, Gudmundsdóttir et al. 1993, Olea et al. 1993), Western blot (WB) (Griffiths et al. 1991), electroimmunotransfer blot (EITB) (Olivier et al. 1992), coagglutination assay (Kimura & Yoshimizu 1981), PCR assay (Miriam et al. 1997, Pascho et al. 1998) and reverse transcription (RT) PCR assay (Cook & Lynch 1999) have been performed. Real-time PCR for *R. salmoninarum* DNA has also been reported by several workers (Powell et al. 2005, Chase et al. 2006, Rhodes et al. 2006). Among them, the FAT, WB, EITB, coagglutination, PCR, and RT-PCR assays are not quantitative. In ELISA and MF-FAT assays the quantitative correlation between culturable or viable cells and the measurement values is unclear. Culture on selective kidney disease medium (SKDM) (Austin et al. 1983) is more relevant and sensitive for the detection of culturable cells (Olivier et al. 1992, Lovely et al. 1994, Griffiths et al. 1996), but long incubation times (12 to 19 wk) are required to detect macroscopic colonies (Benediktsdóttir et al. 1991). This makes the culture technique less practical for BKD diagnostic and screening purposes (Gudmundsdóttir et al. 1993).

Recently, Powell et al. (2005) reported quantification of *msa* gene (Chien et al. 1992) mRNA in the range of 4.8×10^1 to 4.8×10^7 copies in cultured *Renibacterium salmoninarum* cells by real-time RT-PCR. Quantitative assays of *msa* gene mRNA using real-time PCR are expected to indicate the viability of *R. salmoninarum* cells in infected fish for 4 reasons. First, the specificity of the *msa* gene has been confirmed by PCR assay (Brown et al. 1995, McIntosh et al. 1996, Miriam et al. 1997, Chase & Pascho 1998) and RT-PCR assay (Cook & Lynch 1999). Second, bacterial mRNA has a short half-life, measured in minutes (von Gabain et al. 1983, Belasco et al. 1986, Arraiano et al. 1988, Belasco 1993, Alifano et al. 1994, O'Hara et al. 1995, Kushner 1996) due to rapid degradation by the well-organized RNA decay machinery (Rauhut & Klug 1999, Steege 2000); thus, its detection decreases quickly with the loss of viability of bacteria (Sheridan et al. 1998). Third, expression of the *msa* gene is constitutive (Grayson et al. 2002). Finally, the nucleotide sequence of the gene might be conserved among strains because *R. salmoninarum* strains isolated from a wide range of hosts and diverse geographic locations have been similar as determined by serological (Wiens & Kaattari 1989), biochemical (Starliper 1996) and genetic analyses (Grayson et al. 1999).

The purpose of this study were to: (1) develop rapid, sensitive and quantitative methods for detection of *msa* gene mRNA of *Renibacterium salmoninarum* in kidney tissue and ovarian fluid of clinically and subclinically infected fish; (2) elucidate the correlation of *msa* gene mRNA concentration with culturable cell concentration and *msa* gene DNA concentration in chum salmon to determine whether quantitative assays for the mRNA could provide an alternative to culturing to assess the viability of *R. salmoninarum* cells; (3) develop a method to screen for viable *R. salmoninarum* cells in large numbers of ovarian fluid samples in salmonid fish broodstock to prevent vertical transmission of the pathogen; and (4) examine whether quantification of *msa* gene DNA reflects the viability of *R. salmoninarum* cells.

MATERIALS AND METHODS

Bacterial strains and culture. The *Renibacterium salmoninarum* strain Rs-3 used in this study was isolated using KDM-2 agar (Evelyn 1977) from a chum salmon juvenile (120 g) during an outbreak at a wet laboratory at the headquarters of the Hokkaido Fish Hatchery, Eniwa, Hokkaido, Japan in July 2004 and subcultured on KDM-2 agar at 15°C. Stock culture was stored at –80°C in KDM-2 broth supplemented with 15% glycerol. Working cultures were grown on KDM-2 agar at 15°C. Samples of bacterial cultures, kidney tissues and ovarian fluid were cultured at 15°C on SKDM agar (Austin et al. 1983) immediately after collection as described below. Another 7 strains of *R. salmoninarum*, Rs-1, Rs-2, Rs-4, Rs-5, Rs-6, Rs-7 and Rs-8, were identified using nested PCR assay for *msa* gene described below and cultured on KDM-2 agar and in SKDM broth at 15°C. Five strains of *Flavobacterium psychrophilum,* Fp-A, Fp-B, Fp-C, Fp-D, and Fp-E, were identified by PCR targeting 16S rDNA (Toyama et al. 1994) and *gyrB* gene (Izumi & Wakabayashi 2000) and cultured on modified *Cytophaga* agar (Wakabayashi & Egusa 1974) at 15°C. Those strains were isolated in salmonid fish with clinical or subclinical infection including chum salmon, masu salmon, and coho salmon *Oncorhynchus kisutch*, kokanee and rainbow trout *O. mykiss* in Hokkaido, Japan. *Aeromonas salmonicida* ATCC14174 and NCMB1102, *A. hydrophila* NCMB86, *Vibrio anguillarum* NCMB828 and *Pseudomonas fluorescens* NCMB129 were cultured on Bacto™ nutrient agar (Becton Dickinson) at 20°C. *Bacillus subtilis* ATCC6633, *B. cereus* ATCC1178 and *Micrococcus luteus* ATCC9341 were cultured on Bacto™ nutrient agar at 37°C. *Escherichia coli* MV1184 and

JM109 were purchased from a manufacturer (Takara Bio) and cultured on LB agar (tryptone 1%, yeast extract 0.5%, NaCl 0.5%, agar 1.5%, pH 7.2) at 37°C.

Fish. Approximately 5000 fertilized chum salmon eggs were transferred from the Dohoku branch of the Hokkaido Fish Hatchery to a wet laboratory at the headquarters. After hatching the fish were reared in 60 l tanks supplied with well water at 10°C at a constant flow rate of 1.5 l min^{-1} and fed a commercial dry pellet at 2% body weight daily until use. Sixty fish (mean weight, 0.9 g) were collected randomly and kidney tissue samples were spread on trypticase soy agar (TSA; Becton Dickinson), a medium that does not support the growth of *Renibacterium salmoninarum*, and SKDM agar, and subjected to nested PCR assay, real-time PCR assay for *msa* gene mRNA and *msa* gene DNA as described below and the Gram stain reaction; all samples were negative for the 6 assays and, thus, the fish were determined to be free of *R. salmoninarum*.

Detection and quantification of culturable cells. The drop-plate culture method (Evelyn 1977) was performed on bacterial culture, kidney tissue and ovarian fluid samples. Kidney tissue samples were aseptically removed from the sample fish, weighed and homogenized with 9 volumes of a mixture of cold 0.1% peptone and 0.85% NaCl (i.e. peptone–salt, PS) (Evelyn et al. 1981). The bacterial culture, kidney-tissue homogenate and ovarian fluid samples were serially diluted 10-fold to 10^{-6} with cold PS and 3 separate 20 µl drops per dilution were drop-plated onto SKDM agar and TSA and incubated at 15°C for 12 wk. Colony formation was examined weekly for culturable cell numbers (as colony-forming units [CFU]). *Renibacterium salmoninarum* was identified by the Gram stain reaction, nested PCR assay on typical colonies from SKDM agar and by the lack of growth on TSA.

Preparation of *Renibacterium salmoninarum* in bacterial culture. An aliquot of the glycerol stock of *Renibacterium salmoninarum* strain Rs-3 was cultured while shaking in SKDM broth for 7 d at 15°C. A 1 ml aliquot of the culture was added to 1 l of SKDM broth and cultured while shaking for 34 d at 15°C. At 2, 6, 10, 14, 23, 28 and 34 d, 1 ml aliquots were collected for bacterial culture, real-time PCR assay for *msa* gene mRNA and real-time PCR assay for *msa* gene DNA in the manner described below.

Experimental infections. Two types of experimental infections were performed: intraperitoneal (i.p.) injection and bath infection. *Renibacterium salmoninarum* strain Rs-3 was subcultured in KDM-2 broth at 15°C for 7 d. Then the bacterial solution was washed twice with cold PS. The bacterial suspension contained 2.6×10^8 CFU ml^{-1}, 1.7×10^9 *msa* gene mRNA copies ml^{-1}, or 3.6×10^8 *msa* gene DNA copies ml^{-1}. Six groups of 50 chum salmon fry (mean weight, 0.96 g) were placed in separate 60 l tanks. Fish in 5 groups were injected i.p. with 0.1 ml of the bacterial solution and reared for 43 d after infection. One of the 5 groups was observed for cumulative mortality. Another group was injected i.p. with cold PS and used as an uninfected control. The fish were reared at 10°C in the manner described above. At 1, 2, 4 and 6 wk after infection kidney tissue samples were collected from 15 live fish chosen at random; 5 samples were stored for 2 to 3 wk at −80°C for real-time PCR assay for mRNA, 5 were stored for 2 to 3 wk at −80°C for real-time PCR assay for DNA and the other 5 samples were used immediately for bacterial culture. Fifteen of the randomly collected dead fish were tested with the same methodology as described above.

Bath infection was performed because the rapid progress of the infection in i.p. injected fish was clearly not representative of the natural disease course. For bath infection, 5 groups of 100 chum salmon fry (mean weight, 1.64 g) were placed in separate 60 l tanks that contained 20 l still (i.e. no flow through) well water with aeration. Two liters of bacterial culture were prepared and washed twice with cold PS. A 0.5 l aliquot of the bacterial solution was added to each of 4 tanks and kept for 1 h at 10°C with aeration in still water. The bacterial concentration in the 20 l well water at bath infection was 1.0×10^7 CFU ml^{-1}, 1.9×10^7 *msa* gene mRNA copies ml^{-1}, or 5.0×10^6 *msa* gene DNA copies ml^{-1}. One group (100 fry) was observed for determination of cumulative mortality and the 3 other groups were examined by the assays. One-half liter of cold PS was added to the remaining tank, which was used as an uninfected control. Fish were reared for 18 wk at 10°C. At 1, 2, 4, 6, 8, 10, 14 and 18 wk after infection kidney tissue samples were collected from 15 live fish and treated as above.

Collection of ovarian fluid. Wild chum salmon adult females were collected from 8 rivers and reared in 8 hatcheries (Hatcheries D to K) for a few days. Individual ovarian fluid samples (approximately 1 ml per fish) were collected from 30 randomly selected chum salmon adult females from each of the 8 hatcheries where thus far BKD had not been reported. For comparison ovarian fluid samples of 54 kokanee adults reared at Hatchery A and 60 masu salmon adults reared at each of Hatcheries B and C, were also collected. In Hatcheries A, B and C, BKD occurred in adults; daily mortalities, identified by typical granulomatous lesions in the kidney and by the Gram stain reaction of the lesion smears, were 0.16 to 1.09%, 0.01 to 0.13%, and 0.02 to 0.04% in Hatcheries A, B, and C, respectively. Individual samples were divided into 50 µl aliquots and treated as described above; the samples were stored for 2 to 3 d at 4°C before examination. Bacterial culture and nested PCR assays were con-

ducted for each sample. Then the nested PCR positive samples were further used for real-time PCR assay for *msa* gene mRNA and real-time PCR assay for *msa* gene DNA.

Cloning of *msa* gene. Two identical genes encoding MSA (also designated p57), *msa1* and *msa2*, are present in all strains examined thus far (O'Farrell & Strom 1999). A third gene, *msa3*, is present in some strains (Rhodes et al. 2004). The nucleotide sequence of the entire open reading frame (ORF) is the same in all 3 genes. To clone the ORF of the *msa* gene, the complete ORF of *msa1* (O'Farrell & Strom1999) was amplified from *Renibacterium salmoninarum* Rs-3 genomic DNA by PCR using forward primer MSA#1D-For and reverse primer MSA#1B-Rev (Table 1, Wiens et al. 2002). PCR amplification was performed using an ABI 2400 PCR thermal cycler (Applied Biosystems). PCR was performed in a 50 µl reaction mixture containing 1 µl of DNA sample, 50 mM Tris-HCl (pH 9.0), 10 mM KCl, 0.1% Triton-X™ 100, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer and 1.25 units Taq DNA polymerase (Promega). Cycling conditions were preheated at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 1 min at 55°C and extension at 72°C for 2 min, and one additional extension cycle at 72°C for 5 min. Aliquots of PCR products (15 µl) were electrophoresed on a 2% agarose gel containing ethidium bromide at 50 µg $ml^{-1}$and purified by a Band prep™ Kit (GE Healthcare Biosciences), and then ligated into a cloning vector pGEM-T Easy™ (Promega) to form a recombinant plasmid pGEM-T Easy/*msa1* ORF and transformed into *Escherichia coli* MV1184. PCR products of 1379-bp were identified by DNA sequencing with a DYEnamic ET™ terminator Kit (GE Healthcare Biosciences) using an ABI 370 PRISM™ automated DNA sequencer (Applied Biosystems).

***In vitro* transcription of standards.** The recombinant plasmid pGEM-T Easy/*msa1* ORF was linearized with the enzyme *Sph* I and purified with a SepaGene™ Kit (Sanko Jyunyaku). The RNA transcripts were produced using a MEGAscript™ SP6 Kit (Ambion) by priming transcription of the SP6 polymerase-priming site. Transcripts were run on formaldehyde/MOPS gels to confirm the presence of a single band of the correct size. The transcripts were purified with a MEGAclean™ kit (Ambion), and the absence of plasmid DNA in the aliquot was confirmed by nested PCR. RNA yields and purity of the transcripts were determined spectrophotometrically by measuring 260 nm/280 nm absorbance ratios. The concentration of the transcripts was calculated by the molecular weight and Avogádro number, serially diluted 10-fold in Milli-Q™ (Millipore) water (nanopure water) at 5.0 to 5.0×10^9 μl^{-1}, reverse-transcribed and used as standards of real-time PCR assay for the mRNA described below.

cDNA synthesis. cDNA synthesis of standards and unknown samples was conducted using TaqMan™ reverse transcription reagents (Applied Biosystems). The reaction mixture contained 5 µl of 10× RT buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 5.5 mM $MgCl_2$, 0.4 µM dNTP, 20 units RNase inhibitor, 2.5 µM random hexamer primer, 62.5 units Multiscribe™ RTase (Applied Biosystems) and 10 µl of RNA in a 50 µl reaction solution. The reaction solution was incubated at 25°C for 10 min, at 37°C for 60 min, at 95°C for 5 min, held at 4°C and stored at −80°C. cDNA samples of the standard were divided into 10 µl aliquots.

Total RNA extraction of unknown samples. Total RNA was extracted from bacterial cultures, kidney tissue samples and ovarian fluid samples using an RNAqueous™ Kit (Ambion). Briefly, 1 ml of bacterial culture sample was centrifuged at $10\,000 \times g$ for 5 min at 4°C. Supernatants were discarded, 100 µl of RNA storage solution (RNAlater™, Ambion) was added to the pelleted bacterial cells and they were stored at 4°C for 24 h. An approximately 50 mg sample of kidney tissue per fish was collected aseptically, mixed

Table 1. Primers and probe used for quantification of viable *Renibacterium salmoninarum*

Name	Sequence	Position[a]	Orientation	Source
MSA#1D-For	5′-gTC TCC gCT CgT TgC AgA gC-3′	169–188	Forward	Wiens et al. (2002)
MSA#1B-Rev	5′-Cgg CgT TgC CgT CTT ACC-3′	2066–2083	Reverse	Wiens et al. (2002)
UP1	5′-Atg TCg CAA ggT gAA ggg-3′	347–364	Forward	Cook & Lynch (1999)
LP3	5′-TTA CCC gAT CCA gTT CCC-3′	1709–1727	Reverse	Cook & Lynch (1999)
FL7	5′-CgC Agg Agg ACC AgT TgC Ag-3′	472–491	Forward	Miriam et al. (1997)
RL11	5′-ggA gAC TTg CgA TgC gCC-3′	803–820	Reverse	Miriam et al. (1997)
1177F	5′-CCC AgA TAT CCA TgC ACC AgAT-3′	1249–1270	Forward	This study
1313R	5′-CAA CTg AAA Cgg AAC Cag CATT-3′	1364–1385	Reverse	This study
1231T[b]	5′-FAM-Tgg CgA CAA CAC gTA-MGB-3′	1303–1320	Forward	This study

[a]Based on *msa1* gene nucleotide sequence (GenBank accession number AF123890)
[b]TaqMan probe with a 5′-reporter FAM and a 3′-non-fluorescent quencher plus minor groove binder (MGB)

with 100 µl of RNAlater™ and stored at 4°C for 24 h. The kidney samples were centrifuged at 3000 × *g* for 5 min at 4°C and the supernatants were discarded. The samples were then stored at −80°C. Aliquots (50 µl) of ovarian fluid samples were stored at −80°C. The samples of bacterial culture, kidney tissue and ovarian fluid were thawed on ice. One hundred microlitres lysozyme solution containing 1 mg of lysozyme in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) was added to each sample. Total RNA extraction was conducted according to the manufacturer's instructions. DNA in the samples was digested with DNase-I (Turbo DNA-*free*™ Kit, Ambion). Thus, the sample volume was increased 1.2-fold to 60 µl. Finally, the RNA solution contained extracted total RNA from 20 µl of bacterial culture, 1 µl of ovarian fluid or 1 mg of kidney tissue per µl. Then, cDNA synthesis was done as described above.

Real-time PCR assay for *msa* gene mRNA. To quantify cDNA generated by reverse transcription of RNA, real-time PCR with a TaqMan™ probe was conducted using an ABI 7500 real-time PCR system (Applied Biosystems). The 50 µl reaction mixture contained 25 µl of TaqMan™ universal master mix (no uracil-DNA glycosylase [UNG]), 0.9 µM forward primer 1177F, 0.9 µM reverse primer 1313R, 0.4 µM TaqMan™ probe 1231T (Table 1) and 5 µl of cDNA. The TaqMan™ probe consisted of an oligonucleotide with a 5′-reporter fluorescent dye (FAM™, 6-carboxyfluorescein) and a 3′-nonfluoresecnt quencher plus minor groove binder (MGB). Primers 1177F and 1313R and a TaqMan™ probe 1231T were designed using software Primer Express™ ver. 2.0 (Applied Biosystems) based on the nucleotide sequence of the *msa1* gene of *Renibacterium salmoninarum* strain ATCC33209 (GenBank accession number AF123890). Reactions were done in duplicate for each sample. Fifty microlitres of each reaction was added to individual wells of a 96 Microamp™ optical well plate (Applied Biosystems), sealed and placed into the equipment. The signal amplification (ΔRn), which is the normalized reporter signal (i.e. reporter signal minus normalized background), was plotted against the PCR cycle number to generate cycle threshold (C_T) values. The C_T values, which are defined as the PCR cycle number at which an increase in reporter fluorescence above baseline is first detected, were plotted against the log concentration of the calculated copy number of the RNA to give standard curves by linear regression. Variability in the reaction was measured as a square regression coefficient (R^2) after the linear regression describing the relationship between the calculated concentration (log input template) of standard samples and C_T values. The amplification efficiency (*e*) was estimated by the formula $e = 10^{1/s} - 1$, where *s* is the slope. The *e* value can be defined as $X_n = X_0 \times (1 + e)^n$, where X_n is the number of target molecules, and *n* is the number of PCR cycles. Thus, when the amplification efficiency becomes 100%, the *e* value becomes 1.0. The amplification program was 1 cycle of 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. Accumulated data were analyzed using Sequence detector software version 1.2.2 (Applied Biosystems).

DNA extraction. Nucleic acids (DNA and RNA) were extracted from bacterial cells (1 ml of culture fluid), kidney tissue samples (50 mg) from chum salmon fry and ovarian fluid samples (50 µl) from kokanee, masu salmon and chum salmon adults using a SepaGene™ Kit (Sanko Jyunyaku). Briefly, bacterial cultures were centrifuged at 10 000 × *g* for 5 min at 4°C, supernatants were discarded and the pellets were washed twice with cold PS. Finally, nucleic acids were dissolved in Milli-Q™ water at the concentration of nucleic acids derived from 1 ml of bacterial culture or 1 mg of kidney tissue or 1 µl of ovarian fluid per µl. DNA yield and purity were determined spectrophotometrically by measuring 260 nm/280 nm absorbance ratios and 100 to 1000 ng μl^{-1} concentrations.

Real-time PCR assay for *msa* gene DNA. For standards, the recombinant plasmid pGEM-T Easy/*msa1* ORF described above was amplified in *Escherichia coli* MV1184 and extracted by a FlexiPrep™ Kit (GE Healthcare Biosciences). The concentration of the extracted plasmid was calculated, 10-fold serially diluted with Milli-Q™ water at 5.0-5.0 × 10^9 copies μl^{-1} and used as a standard.

Specificity of primers and probe for real-time PCR. DNA was extracted from suspensions of 8 *Renibacterium salmoninarum* strains and each of the other bacterial strains (see text) in PBS (approximately 10^6 to 10^8 CFU ml^{-1}) by SepaGene™ Kit. Ovarian fluid, kidney tissue, gill tissue and skin tissue samples of 5 fish each from kokanee, chum and masu salmon were collected. DNA extracted from those tissue samples were confirmed negative by nested PCR targeting *msa* gene as described above, which failed to yield a specific 349 bp product. The extracted DNA was used as a template in real-time PCR for *msa* gene DNA.

Screening of ovarian fluid by nested PCR assay. Ovarian fluid sampled from each fish was divided into 3 aliquots of 100 µl each. An aliquot was cultured immediately after collection and other aliquots were stored at −80°C for the assays as described above. To screen large numbers of ovarian fluid samples a nested PCR assay was performed and the positive samples were further examined by real-time PCR assays for the mRNA and DNA. Two sets of primers were used. For the first round PCR, primers UP-1 and LP-3, which were reported by Cook & Lynch (1999) to amplify the 1379 bp partial *msa1* gene, and for second-round PCR,

primers FL7 and RL11, which were reported by Miriam et al. (1997) to amplify 349 bp (Table 1) were used. The PCR assay was conducted as described above. The annealing temperature was 55°C for the first-round PCR and 60°C for the second-round PCR. After the first PCR, 1 µl of the reaction mixture was added to the nested PCR reaction. A 15 µl aliquot of the nested PCR reaction mixture was electrophoresed at 100 V for 30 min in 2% agarose gels containing ethidium bromide at 0.5 µg ml^{-1}. An All-Purpose Hi-Lo™ DNA marker (Bionexus,) was used as a DNA size marker. Milli-Q™ water was used as a negative control. The recombinant plasmid pGEM-T Easy/*msa1* ORF was used as a positive control.

Sensitivity of nested PCR assay of seeded kidney tissue and ovarian fluid. *Renibacterium salmoninarum* strain Rs-3 was subcultured in KDM-2 broth at 15°C for 1 wk. The culturable cell concentration in a culture was determined as described above to be 5.0×10^8 CFU ml^{-1}. The culture was serially diluted 10-fold with PS to 10^{-8}. Kidney tissue samples were removed from chum salmon female adults in Hatchery G, homogenized and stored at −80°C until use. Ovarian fluid samples of chum salmon in Hatchery G were collected as described above. The kidney tissues and ovarian fluid samples were examined by nested PCR assay and negative samples were used for the assays. Serial dilutions of the bacteria (100 µl) were added to 50 mg homogenized kidney tissue or 50 µl ovarian fluid, mixed and incubated at 15°C for 1 h. Then, DNA was extracted using a SepaGene™ Kit, dissolved in 50 µl water and used to determine the sensitivity of the nested PCR assay.

Correlation analyses. The square regression coefficient (R^2) after linear regression was used to measure the relationship between the CFU concentration and the *msa* gene mRNA or *msa* gene DNA concentration. Linear regression and the square regression coefficient were analyzed using Microsoft Excel 2003™ (Microsoft).

RESULTS

Specificity of primers and probe for real-time PCR assay

The specificity of the primers 1177F and 1313R, and a TaqMan™ probe 1231T was confirmed. Approximately 10^6 to 10^7 copies of *msa* gene DNA $µl^{-1}$ were detected from DNA of all 8 *Renibacterium salmoninarum* strains tested, whereas no copy was detected from DNA of both the other bacterial strain samples including fish pathogens and the fish tissue samples (see 'Materials and methods').

Reverse transcription followed by real-time PCR assay for *msa* gene mRNA

The ΔRn of the standards with 10-fold serial dilutions ranging from 5.0×10^1 to 5.0×10^9 *msa* RNA $µl^{-1}$ were plotted against PCR cycle numbers (Fig. 1A). C_T values plotted against the standard samples (log input RNA) gave a straight line (Fig. 1B). The R^2 value was 0.999, which showed a high correlation. The slope of the reaction was −3.356, and the amplification efficiency (*e*) was 0.907. The detection limit was 5.0×10^1 copies $µl^{-1}$, corresponding to 3.0×10^2 copies mg^{-1} of kidney tissue samples or $µl^{-1}$ of ovarian fluid samples because samples were diluted 1.2-fold in DNase I-treatment before cDNA synthesis and further diluted 5-fold in cDNA synthesis.

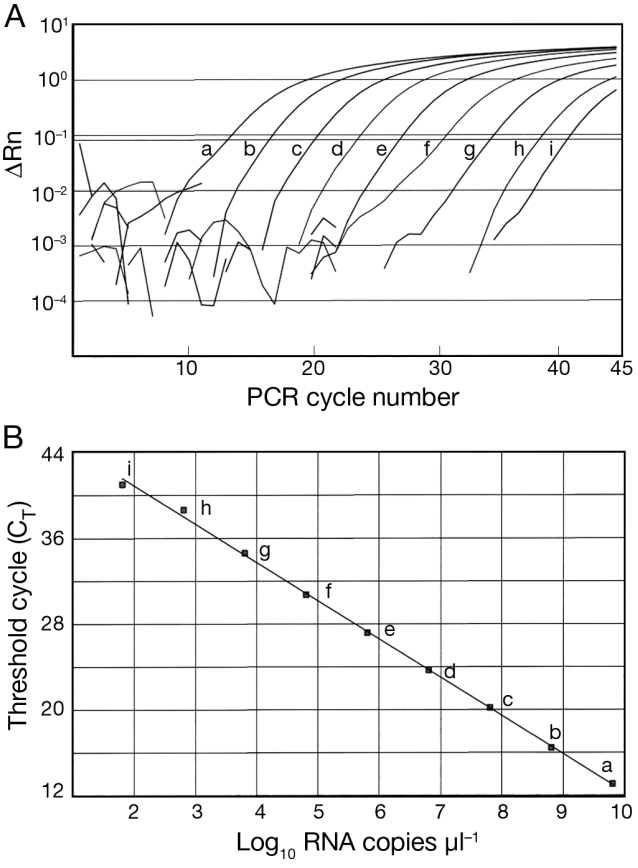


Fig. 1. Reverse transcription followed by real-time PCR assay for *msa* gene RNA. *In vitro*-transcribed *msa* gene RNA with 10-fold serial dilutions in Milli-Q™ water (nanopure water) was reverse transcribed and used as a standard for real-time PCR assay. (A) Amplification plots of ΔRn versus PCR cycle number. Each plot contains one sample replica. (B) Standard curve of threshold PCR cycle number (C_T) versus log concentration of *in vitro* transcripts followed by reverse transcription (cDNA of *msa* gene RNA) in each standard. a, 5.0×10^9 *msa* RNA copies $µl^{-1}$; b, 5.0×10^8; c, 5.0×10^7; d, 5.0×10^6; e, 5.0×10^5; f, 5.0×10^4; g, 5.0×10^3; h, 5.0×10^2; i, 5.0×10^1

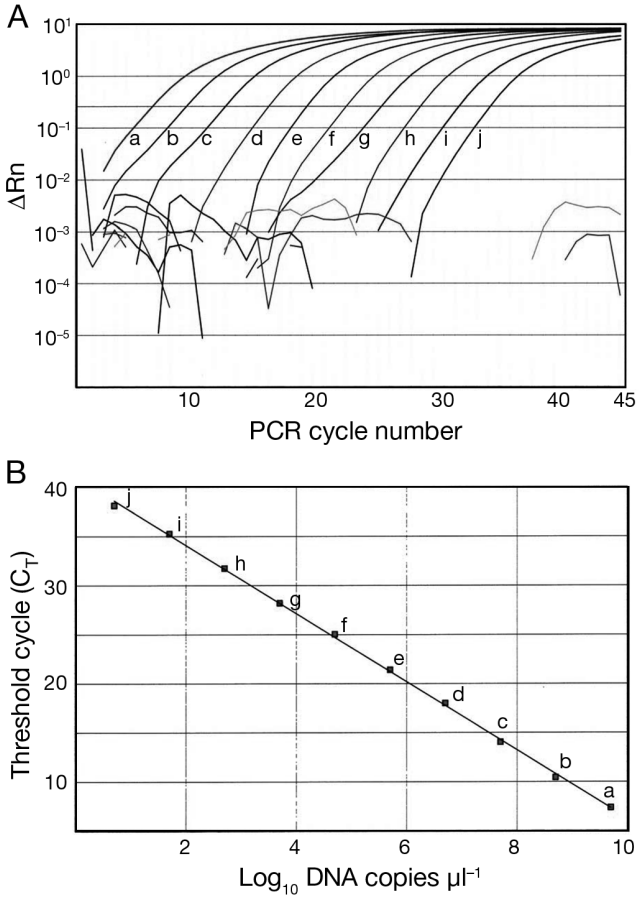


Fig. 2. Real-time PCR assay for *msa* gene DNA. A recombinant plasmid pGEM-T Easy™/*msa1* ORF, which encodes the complete *msa1* gene ORF, was serially diluted 10-fold with Milli-Q™ water and used as a standard. (A) Amplification plots of ΔRn versus PCR cycle number. Each plot contains one sample replica. (B) Standard curve of threshold PCR cycle number (C_T) versus log concentration of the recombinant plasmid in each standard. a, 5.0×10^9 *msa* gene DNA copies µl^{-1}; b, 5.0×10^8; c, 5.0×10^7; d, 5.0×10^6; e, 5.0×10^5; f, 5.0×10^4; g, 5.0×10^3; h, 5.0×10^2; i, 5.0×10^1; j, 5.0

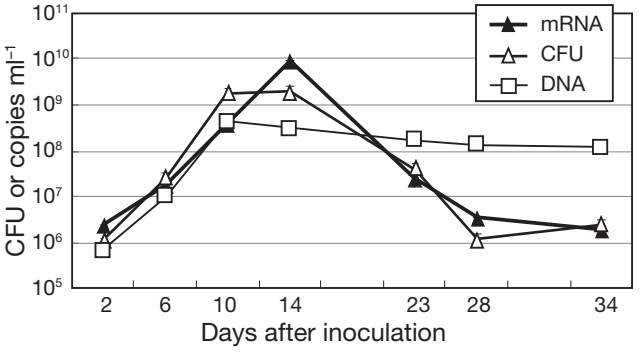


Fig. 3. Comparison between concentrations of *msa* gene mRNA, *msa* gene DNA, and culturable *Renibacterium salmoninarum* cells in bacterial culture. DNA and mRNA were quantified by real-time PCR assay and reverse transcription followed by real-time PCR assay, respectively. The culturable cell concentration was determined by drop-plate culture on SKDM agar plates for 12 wk as CFU. The cells were incubated in SKDM broth while shaking for 34 d at 15°C

Real-time PCR assay for *msa* gene DNA

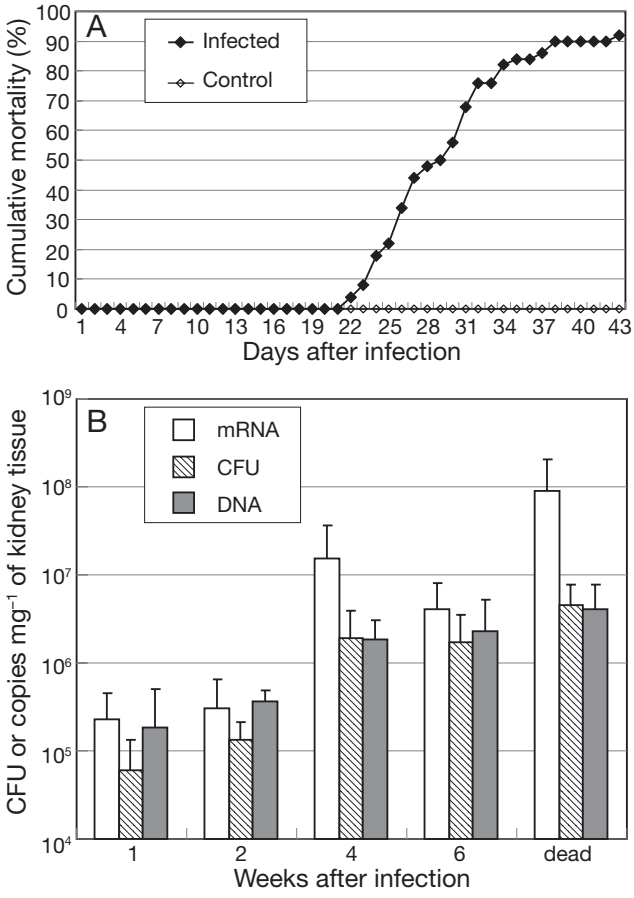
The ΔRn of the standards with 10-fold serial dilutions ranging from 5.0 to 5.0×10^9 *msa* gene DNA µl^{-1} were plotted against PCR cycle numbers (Fig. 2A). C_T values plotted against the standard samples (log input DNA) gave a straight line at 5.0 to 5.0×10^9 copies µl^{-1} (Fig. 2B). The R^2 value was 0.999, which showed a high correlation. The slope of the reaction was –3.474, and the amplification efficiency was 0.940. The detection limit was 5 copies µl^{-1}, corresponding to 5 copies mg^{-1} of kidney tissue or µl^{-1} of ovarian fluid. The detection limit of *msa* gene DNA was 60-fold higher than that of *msa* gene mRNA.

Bacterial culture

The *msa* gene mRNA concentration on Day 0 was 3.3×10^6 ml^{-1}(mean), increased to 9.2×10^9 ml^{-1} on Day 14, and decreased to 2.4×10^7, 3.3×10^6, and 1.9×10^6 ml^{-1}, respectively, on Days 23, 28, and 34 (Fig. 3). The changes in concentrations of culturable cells showed similar patterns. In contrast, the concentration of *msa* gene DNA was 1.2×10^6 ml^{-1} (mean) on Day 0, increased to 4.2×10^8 on Day 10 and remained constant on Days 14 to 34. The concentration of *msa* mRNA plotted against culturable cell concentration in samples gave a linear regression. The R^2 value was 0.888 and showed a strong relationship between the 2 values. No inhibitory effect on real-time PCR assays was observed in the samples. No contamination was observed on SKDM agar inoculated with the samples.

Infectious experiment I: intraperitoneal injection

Chum salmon fry (mean weight, 0.96 g) began to die 21 d after infection. The cumulative mortality reached 92% on Day 43 after infection (Fig. 4A). The cumulative mortality of uninfected controls was 0%. Approximately half of the infected fish showed clinical signs that included darkening of the body color and cessation of feeding. Dead fish showed some or all of the clinical signs including pale gills, swollen abdomens due to ascites, exophthalmos, a pale and swollen kidney, a pale liver, a viscous yellow fluid in the intestine and the presence of culturable *Renibacterium salmoninarum* cells on SKDM agar plates in kidney tissue. Live fish collected at 4 and 6 wk after infection showed clin-



ical signs similar to those of the dead fish. All the smears of the kidney tissue showed Gram-positive diplococcobacilli.

In live fish samples the concentrations of mRNA were 2.3×10^5 and 3.1×10^5 mg^{-1} of kidney tissue (mean), respectively, at Week 1 and Week 2 after infection and increased to 1.5×10^7 and 4.1×10^6, respectively, at Week 4 and Week 6 (Fig. 4B, Table 2), which were similar to levels in dead fish samples. The culturable cell concentrations and the *msa* gene DNA concentration showed similar patterns to the mRNA and culturable *Renibacterium salmoninarum* cell concentrations throughout the experimental period (1 to 6 wk after infection). The prevalence and levels of *R. salmoninarum* determined by the 3 assays were similar throughout the experimental period (Table 2). The beginning of mortality coincided with the increases in mRNA, DNA and culturable cell concentrations (Fig. 4A,B).

Fig. 4. Correlation between *msa* gene mRNA concentration in chum salmon fry kidney injected i.p. with *Renibacterium salmoninarum*, *msa* gene DNA concentration and CFU concentration. The fish were observed for 43 d (6 wk) after infection. (A) Cumulative mortality (%) in infected fish (◆) and uninfected control fish (◇). (B) Comparison of the mRNA, gene DNA and CFU concentrations in kidney tissue of infected fish. Live fish were collected 1, 2, 4 and 6 wk after infection. Dead fish were collected during the experimental period. Bars show mean values + SD

Table 2. Culturable cell concentration, *msa* gene mRNA concentration and *msa* gene DNA concentration in chum salmon fry kidney tissue experimentally challenged with *Renibacterium salmoninarum* by i.p. injection and bath infection

Challenge method	Weeks after infection	Status of sample fish	Culturable cells[a] CFU mg^{-1} of kidney[d]	Prevalence[e]	*msa* gene mRNA[b] Copies mg^{-1} of kidney[d]	Prevalence[e]	*msa* gene DNA[c] Copies mg^{-1} of kidney[d]	Prevalence[e]
Intraperitoneal injection	1	Live	$1.5 - 1.7 \times 10^5$	5/5	$3.5 \times 10^4 - 5.3 \times 10^5$	4/5	$2.0 \times 10^3 - 7.3 \times 10^5$	4/5
	2	Live	$9.2 \times 10^3 - 2.0 \times 10^5$	5/5	$3.0 \times 10^4 - 7.7 \times 10^5$	4/5	$2.2 \times 10^5 - 5.1 \times 10^5$	5/5
	4	Live	$2.3 \times 10^5 - 5.0 \times 10^6$	5/5	$8.0 \times 10^5 - 5.3 \times 10^7$	5/5	$1.7 \times 10^5 - 2.9 \times 10^6$	5/5
	6	Live	$2.5 \times 10^5 - 3.6 \times 10^6$	5/5	$2.3 \times 10^5 - 8.5 \times 10^6$	4/5	$7.0 \times 10^3 - 6.4 \times 10^6$	5/5
	–	Dead	$2.0 \times 10^6 - 1.6 \times 10^7$	5/5	$1.6 \times 10^7 - 2.9 \times 10^8$	5/5	$1.2 \times 10^5 - 1.0 \times 10^7$	5/5
Bath infection	1	Live	< 0.5	0/5	$1.4 \times 10^4 - 3.6 \times 0^4$	2/5	$< 5.0 \times 10^1$	0/5
	2	Live	$0.7 - 1.2$	2/5	$< 3.0 \times 10^2$	0/5	$< 5.0 \times 10^1$	0/5
	4	Live	$0.8 - 1.5 \times 10^1$	4/5	8.0×10^4	1/5	7.1	1/5
	6	Live	$1.2 - 7.5$	3/5	8.7×10^3	1/5	$< 5.0 \times 10^1$	0/5
	8	Live	$1.3 \times 10^1 - 2.7 \times 10^1$	2/5	$4.1 \times 10^2 - 7.8 \times 10^2$	3/5	8.3×10^2	1/5
	10	Live	$1.0 \times 10^1 - 6.3 \times 10^1$	4/5	$4.6 \times 10^2 - 2.0 \times 10^3$	4/5	$< 5.0 \times 10^1$	0/5
	14	Live	$1.4 \times 10^2 - 5.7 \times 10^4$	4/5	$8.2 \times 10^2 - 1.6 \times 10^5$	4/5	$3.5 \times 10^2 - 9.0 \times 10^4$	4/5
	18	Live	$5.3 \times 10^1 - 1.8 \times 10^7$	5/5	$8.4 \times 10^2 - 3.3 \times 10^3$	5/5	$2.9 \times 10^2 - 4.0 \times 10^5$	4/5
	–	Dead	$2.5 \times 10^5 - 1.5 \times 10^8$	5/5	$2.9 \times 10^5 - 2.6 \times 10^6$	4/5	$6.1 \times 10^3 - 2.4 \times 10^4$	5/5

[a]CFU concentration was determined by drop-plate culture (Evelyn et al. 1977) on SKDM agar (Austin et al. 1983) for 12 wk. The detection limit is 0.5 CFU mg^{-1} of kidney tissue

[b]*msa* gene mRNA concentration was determined by reverse transcription followed by real-time PCR assay. The detection limit is 3×10^2 copies mg^{-1} of kidney tissue

[c]*msa* gene DNA concentration was determined by real-time PCR assay. The detection limit is 5×10^1 copies mg^{-1} of kidney tissue

[d]Figures indicate range of the values of every sample

[e]Prevalence indicates number of positive samples per number of samples examined

Lower or no concentration values of DNA, approximately one-tenth of the actual values or lower, were obtained in real-time PCR assay for the DNA, revealing an inhibitory effect by kidney tissue samples (data not shown). Thus, the DNA samples of the kidney tissue were diluted 10-fold with Milli-Q™ water and used for the assay, resulting in decrease of the detection limit of the DNA quantification to 50 copies mg^{-1} of kidney tissue, whereas no inhibitory effect was observed in real-time PCR assay for the mRNA. No contamination was observed in bacterial cultures on SKDM agar.

The concentration of *msa* mRNA plotted against culturable cell concentration in samples gave a linear regression; the R^2 value was 0.924 and highly correlated, whereas the R^2 value between culturable cell concentration and the DNA concentration was 0.992 and, thus, showed a higher correlation.

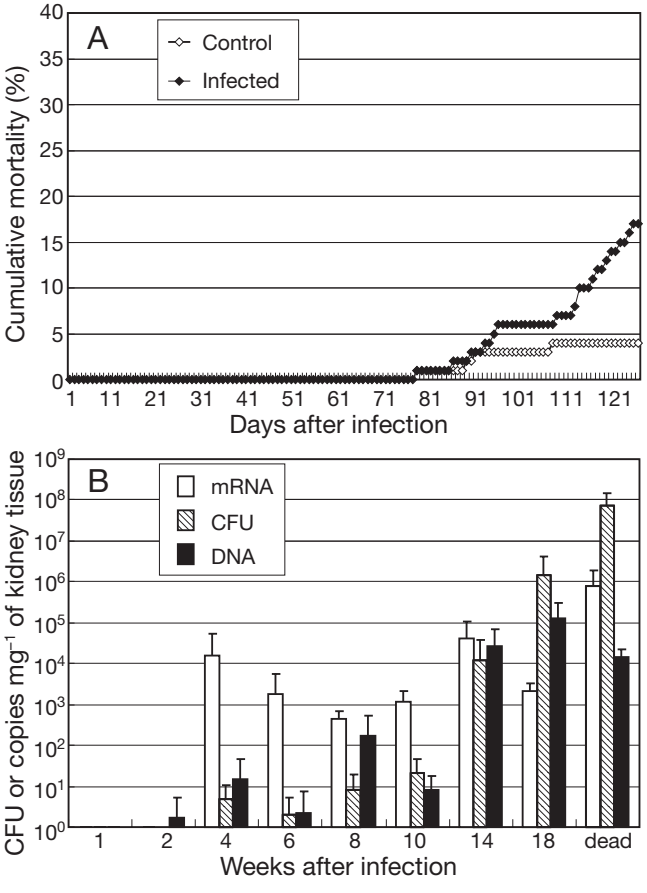


Fig. 5. Correlation of *msa* gene mRNA concentration in chum salmon fry kidney infected with *Renibacterium salmoninarum* in bath infection with *msa* gene DNA and CFU concentrations. The fish were observed for 126 d (18 wk) after infection. (A) Cumulative mortality (%) in infected fish and uninfected control fish. (B) Comparison among mRNA, gene DNA and CFU concentrations in infected fish. Live fish were collected 1, 2, 4, 6, 8, 10, 14 and 18 wk after infection. Dead fish were collected during the experimental period. Bars show mean values +SD

Infectious experiment II: bath infection

Infected chum salmon fry (mean weight, 1.64 g) began to die on Day 77 after infection. Deaths continued and the cumulative mortality on Day 127 was 17%. (Fig. 5A). The cumulative mortality of the uninfected control was 4%. Most dead fish (31 of 42 fish collected) and some live fish collected (15 of 120 fish) showed white granulomatous lesions in the kidney. Approximately 90% of the dead fish showed clinical signs that included darkening of the skin color, pale gills, exophthalmos, a pale liver, a pale and swollen kidney and a yellow viscous fluid in the intestine. Live fish collected 1 to 10 wk after infection showed no clinical signs except for pale gills and darkening of the skin color in approximately 10% of the fish, whereas those collected 14 and 18 wk after infection showed clinical signs similar to those of the dead fish.

The concentration of *msa* gene mRNA was higher in live fish collected in the early phase of bath infection (4 to 10 wk after infection) compared with the DNA and culturable cell concentrations at corresponding sampling times (Fig. 5B, Table 2), whereas the DNA and culturable cell concentrations increased gradually 1 to 10 wk after infection to reach the levels similar to those of the dead fish 14 and 18 wk after infection (Fig. 5B, Table 2). In the early phase of infection (1 to 10 wk after infection) the prevalence of *Renibacterium salmoninarum* determined by the mRNA quantification was higher than that determined by the DNA quantification, particularly in the samples collected during the early phase of bath infection (Table 2), while in the late phase of infection (14 and 18 wk after infection and in dead fish) the prevalence of *R. salmoninarum* determined by the 3 assays was almost the same. The beginning of death coincided with increases in the mRNA, the DNA and CFU concentrations (Fig. 5A,B).

Lower DNA concentrations, approximately one-tenth of the actual values obtained in real-time PCR assay, revealed the inhibitory effect of kidney tissue samples (data not shown). Therefore, the DNA samples of the kidney tissue were diluted 10-fold with Milli-Q™ water and used for the assay resulting in a decrease in the detection limit to 50 copies mg^{-1} of kidney tissue, whereas no inhibitory effect was observed in real-time PCR assay for the mRNA. No contamination was observed on SKDM agar inoculated with the samples.

The samples gave a linear regression between the culturable cell and mRNA concentrations; the R^2 value

was 0.502 and showed a weak relationship between the 2 values, whereas the R^2 value between the culturable cell and DNA concentrations was 0.813 and showed a higher correlation.

Sensitivity of nested PCR assay for seeded kidney tissue and ovarian fluid samples

Nested PCR amplification 349 bp products were obtained from DNA extracts of 1.0×10^1 to 1.0×10^3 CFU μl^{-1} of bacterial culture (Fig. 6A), 1.0×10^1 to 1.0×10^3 CFU mg^{-1} of kidney tissue (Fig. 6B), and 1.0×10^1 to 1.0×10^3 CFU μl^{-1} of ovarian fluid (Fig. 6C). No inhibitory effect by the tissue was observed.

Ovarian fluid sample of salmonids

Ovarian fluid samples collected from chum salmon were 0 to 6.7% positive in culture. Detection rates (number positive / number examined) were 0 to 30% in nested PCR assay, 0 to 30% in real-time PCR assay for mRNA and 6.7 to 30% in real-time PCR assay for DNA (Table 3, Fig. 7A), whereas prevalence from masu salmon and kokanee was 3.3 to 11% positive in culture; detection rates were 25 to 44.4% in nested PCR, 16.7 to 31.5% in real-time PCR assay for mRNA, and 25 to 44.4% in real-time PCR assay for DNA. The prevalence of culturable cells was lower than that of DNA and mRNA in all 3 salmonid species. Contamination with various faster growing bacteria was observed on SKDM agar plates inoculated with lower dilutions (10^0 to 10^{-2} dilution) of ovarian fluid samples of salmonid fish, in particular chum salmon (Table 3), whereas higher dilutions (10^{-3} to 10^{-6}) produced neither contamination nor *Renibacterium salmoninarum* colonies.

The concentrations of mRNA and DNA in chum salmon ovarian fluid were 9.7×10^2 to 2.5×10^4 μl^{-1} (mean) and 2.8×10^2 to 1.1×10^3 μl^{-1} (mean), respectively (Table 3, Fig. 7B). The masu salmon samples showed similar results, whereas the kokanee samples showed higher values.

The R^2 value between the mRNA concentration (mean) and detection rate of the mRNA was 0.590 and weakly correlated, while the R^2 value between DNA concentration and its detection rate was 0.716.

DISCUSSION

In the reverse transcription (RT) followed by real-time PCR assay for *msa* gene mRNA the quantifiable range was 5.0×10^1 to 5.0×10^9 copies μl^{-1} of sample, which corresponds to 3×10^2 to 3×10^{10} copies mg^{-1} of kidney tissue or μl^{-1} of ovarian fluid. The square

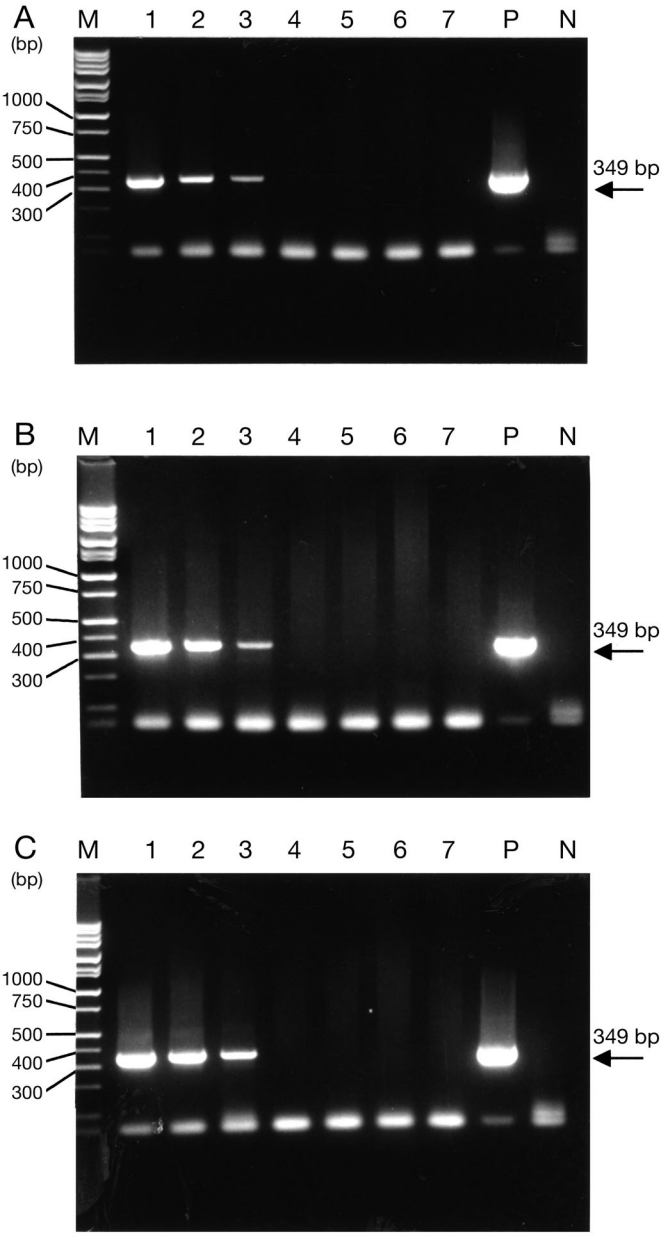


Fig. 6. Sensitivity of nested PCR assay for bacterial culture, seeded kidney tissue homogenate and seeded ovarian fluid samples. PCR amplification products were electrophoresed on 2% agarose gels containing ethidium bromide at 0.5 $\mu g\ ml^{-1}$. (A) Nested PCR products from DNA extract of *Renibacterium salmoninarum* cells serially diluted 10-fold in bacterial culture. (B) Nested PCR products from DNA extract of kidney homogenate samples seeded with *R. salmoninarum* cells serially diluted 10-fold. (C) Nested PCR products from DNA extract of ovarian fluid samples seeded with *R. salmoninarum* cells serially diluted 10-fold. Lane 1, 1.0×10^3 CFU μl^{-1}; Lane 2, 1.0×10^2 CFU μl^{-1}; Lane 3, 1.0×10^1 CFU μl^{-1}; Lane 4, 1.0×1 CFU μl^{-1}; Lane 5, 1.0×10^{-1} CFU μl^{-1}; Lane 6, 1.0×10^{-2} CFU μl^{-1}; Lane 7, unseeded sample; M, DNA marker (Hi-Lo™ All purpose DNA marker, Bionexus); P, Plasmid pGEM-T Easy™/*msa1* ORF as a positive control; N, Milli-Q™ water (nanopure water) as a negative control. Arrows indicate the position of the 349 bp PCR product

Table 3. Detection of *Renibacterium salmoninarum* in clinically and subclinically infected salmonid ovarian fluid

Species	Site	Sampling Date	n	BKD[a] daily mortality	Colony[b] Culture positive	Culture negative	Contaminated	Prevalence[e]	Nested PCR prevalence[e]	Real-time PCR for mRNA[c] % positive of no. tested	Copies μl^{-1}[f]	Real-time PCR for DNA[d] % positive of no. tested	Copies μl^{-1}[f]
Kokanee	A	Sep 2004	54	0.16–1.09%	10	16	28	11.1%	44.4%	31.5%	2.2×10^{3}–4×10^{6}	44.4%	5.3–1.6×10^{6}
Masu	B	Sep 2005	60	0.01–0.13%	7	20	33	3.3%	26.7%	16.7%	4.9×10^{2}–9×10^{3}	26.7%	1.1×10^{1}–1.1×10^{3}
salmon	C	Sep 2005	60	0.02–0.04%	8	32	20	3.3%	25%	18.3%	3.2×10^{2}–5.9×10^{4}	25%	2.6×10^{1}–1.6×10^{3}
Chum	D	Sep 2005	30	None	2	4	24	6.7%	6.7%	0%	$<3.0\times10^{2}$	6.7%	2.0×10^{1}–3.5×10^{1}
salmon	E	Oct 2005	30	None	-	6	24	0%	16.7%	13.3%	6.6×10^{2}–4.1×10^{3}	16.7%	1.9×10^{1}–1.1×10^{2}
	F	Oct 2005	30	None	-	13	17	0%	30%	30%	1.3×10^{3}–1.7×10^{4}	30%	1.1×10^{1}–5.7×10^{1}
	G	Oct 2005	30	None	-	10	20	0%	0%	Not done	Not done	Not done	Not done
	H	Oct 2005	30	None	-	4	26	0%	26.7%	16.7%	5.1×10^{2}–1.2×10^{5}	26.7%	1.6×10^{1}–8.5×10^{1}
	I	Oct 2005	30	None	-	-	30	0%	16.7%	10%	3.8×10^{2}–1.5×10^{3}	16.7%	1.1×10^{1}–1.2×10^{2}
	J	Oct 2005	30	None	-	-	30	0%	10%	10%	1.1×10^{3}–7.6×10^{3}	10%	1.4×10^{1}–3.4×10^{1}
	K	Oct 2005	30	None	-	-	30	0%	6.7%	6.7%	8.5×10^{2}–2.9×10^{3}	6.7%	4.9×10^{1}–8.1×10^{1}

[a]All dead fish kidneys were examined by clinical signs, Gram stain reaction, and nested PCR assay for *msa* gene DNA and confirmed to be BKD
[b]Culturable cells were detected by drop-plate culture (Evelyn 1977) on SKDM medium (Austin et al. 1983) for 12 wk. Culture-positive, culture-negative, and contaminated sample numbers are indicated. Detection limit is 1 CFU per 20 µl of ovarian fluid
[c]Only nested-PCR positive samples were examined by reverse transcription followed by real-time PCR assay for *msa* gene mRNA. Detection limit is 3.0×10^{2} copies μl^{-1} of ovarian fluid
[d]Only nested-PCR positive samples were examined for real-time PCR assay for *msa* gene DNA. Detection limit is 5 copies μl^{-1} of ovarian fluid
[e]Prevalence indicates percentage of positive samples in samples examined
[f]Figures indicate range of values of every sample

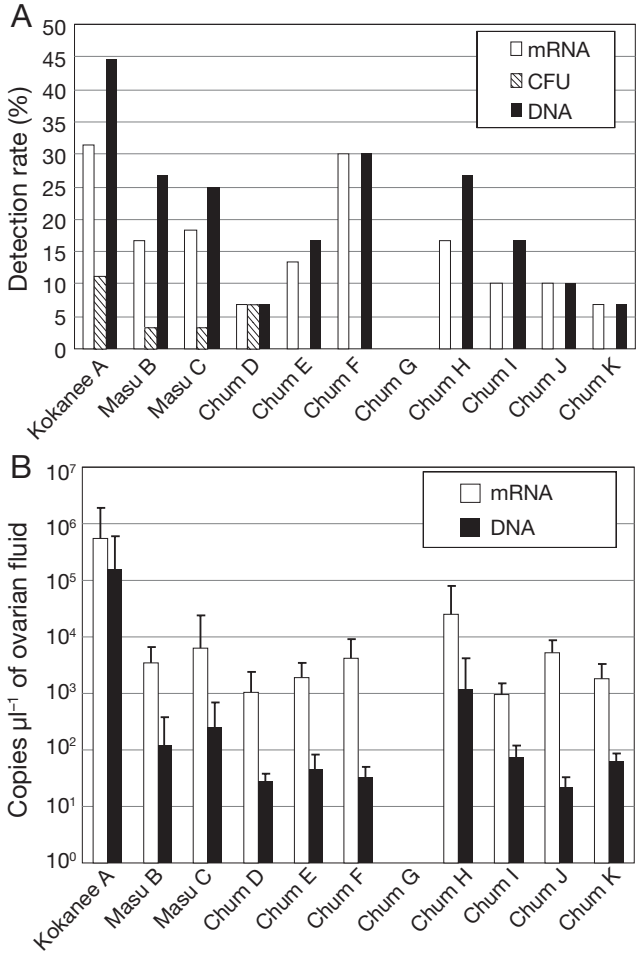


Fig. 7. Detection rate and quantification of *msa* gene mRNA, CFU, and *msa* gene DNA in ovarian fluid samples of adult kokanee and masu and chum salmon. (A) Detection rate of CFU, *msa* gene mRNA and *msa* gene DNA in ovarian fluid samples from kokanee adults reared at 1 hatchery, masu salmon adults reared at 2 hatcheries and wild chum salmon adults collected from 8 rivers and reared in 8 hatcheries for a few days. CFU, mRNA and DNA were detected, respectively, by drop-plate culture on SKDM agar for 12 wk, reverse transcription followed by real-time PCR, and real-time PCR. (B) Quantification of mRNA and DNA in ovarian fluid. Ovarian fluid samples were screened by nested PCR assay and the positive samples were used for real-time PCR assay for DNA and reverse transcription followed by real-time PCR assay for mRNA. Bars show mean values + SD

regression coefficient (R^2) and amplification efficiency (*e*) in the standard were 0.999 and 0.904, respectively. The linearity of the standard curves with a range at 8 log units, high R^2 value and high *e* value confirmed that the assay would be suited for quantitative measurement of *msa* gene mRNA. The linear relationship and detection limit were higher than those of other reports using a quantitative PCR assay for *msa* gene mRNA of *Renibacterium salmoninarum* (Powell et al. 2005). The detection limit of *msa* gene mRNA in this

study was one log unit lower than that of *msa* gene DNA. Thus, the difference is due to the efficiency of reverse transcription in the mRNA quantification, which is approximately 10%. However, in this study total extraction and cDNA synthesis of unknown samples were done under the same conditions and, thus, the reverse-transcription efficiency did not influence the overall pattern of the results.

In bacterial culture and in kidney tissue of chum salmon fry infected by i.p. injection and in the late phase of bath infection (14 and 18 wk after infection), in which fish showed clinical signs and the culturable cell concentration of kidney tissue was more than 10^4 CFU mg^{-1}, the *msa* gene mRNA concentration correlated well with the culturable *Renibacterium salmoninarum* cell concentration. Moreover, prevalence of mRNA was similar to that of culturable cells. Thus, detection and quantification of *msa* gene mRNA would reflect the infection status of the fish. While in the early phase of bath infection (1 to 10 wk after infection) in which chum salmon fry showed no clinical signs, higher mRNA concentrations in kidney tissue samples were detected in comparison with the culturable cell concentration ($<10^4$ CFU mg^{-1} of kidney tissue). This resulted in a weaker correlation between the 2 values in bath infection ($R^2 = 0.502$). The higher mRNA concentration and higher detection rates in such fish would reflect the viability of *R. salmoninarum* cells. In kidney tissue of both clinically and subclinically infected fish real-time PCR assay for the mRNA would be rapid (approximately 30 h compared with 12 wk or more in culture) and accurate, although it would be less sensitive than culture on SKDM agar plates, especially during the early phase of the bath infection.

The ability of quantification of *msa* gene mRNA by real-time PCR to assess the viability of *Renibacterium salmoninarum* cells would be supported by the following 4 reasons. First, the primers and Taqman™ probe used in this study were specific to *R. salmoninarum*. The specificity of the nucleotide sequence of *msa* gene has been demonstrated by this study and also by PCR assay (Brown et al. 1995, McIntosh et al. 1996, Miriam et al. 1997, Chase & Pascho 1998) and RT-PCR assay (Cook & Lynch 1999). Second, compared with bacterial gene DNA and rRNA, mRNA has a short half-life, measured in minutes, and would be a good indicator of cell viability (Arraiano et al. 1988, Belasco 1993, Alifano et al. 1994), although the persistence of mRNA may vary from a few minutes to several hours depending on the environmental conditions and mRNA from different species (Sheridan et al. 1998, McIngvale et al. 2002), and the methods of inactivating treatments and subsequent holding conditions (Jou et al. 1997, Sheridan et al. 1998, Birch et al. 2001). Cook & Lynch (1999) reported that inactivation of cultured *R. salmoninarum* by rifampicin or erythromycin produced a loss of *msa* gene mRNA detection by nested RT-PCR corresponding to a loss of culturable *R. salmoninarum* concentration and that nested RT-PCR identified similar levels of infected fish in subclinical live Atlantic salmon *Salmo salar* as determined by culture on SKDM agar. Third, *msa* gene is constitutively expressed (Grayson et al. 2002) and thus, would enable us to detect viable *R. salmoninarum* cells irrespective of the status of the bacteria. Finally, nucleotide sequences of the *msa* gene ORF might be conserved among strains because the strains isolated from a wide range of hosts and geographic areas have indicated relatively low genetic diversity based on serological (Wiens & Kaattari 1989), biochemical (Starliper 1996) and genetic data (Grayson et al. 1999). Thus, the primers and TaqMan™ probe used in this study would be valid to detect and quantify *msa* gene DNA and *msa* gene mRNA of *R. salmoninarum* strains isolated in salmonid fish in Hokkaido, Japan.

In ovarian fluid samples from clinically and subclinically infected salmonid broodstock, quantification of *msa* gene mRNA would be a good indicator of viability of *Renibacterium salmoninarum* cells. Due to the contamination of SKDM agar with other faster-growing microorganisms the correlation between culturable *R. salmoninarum* and mRNA concentrations in ovarian fluid was unclear. However, the salmonid ovarian fluid samples positive for real-time PCR assay for mRNA might have contained lower concentrations of the culturable cells, which were at most 5.0×10^1 CFU μl^{-1}, corresponding to 1.0×10^2 *msa* gene DNA μl^{-1}. Contamination was observed only on SKDM agar plates inoculated with 10^0 to 10^{-2} dilutions and neither contamination nor *R. salmoninarum* cell colonies were observed in those inoculated with higher dilutions (10^{-3} to 10^{-6}), thus, the detection limit of 10^{-3} dilutions was 5×10^1 CFU μl^{-1}. The prevalence and quantification of *msa* gene DNA and *msa* gene mRNA support this idea. Viable but nonculturable *R. salmoninarum* cells might contribute to the detection and quantification of mRNA in ovarian fluid samples, although the presence of such cells in *R. salmoninarum* has not been reported thus far. The quantification of mRNA concentration (mean) correlated with the detection rate ($R^2 = 0.590$) reflect the viability of *R. salmoninarum* in clinically and subclinically infected broodstock.

Contamination with other faster-growing microorganisms occurred in 28 of 54 samples examined, 33 of 60, and 20 of 60, and 17 of 30 to 30 of 30, respectively, in kokanee, masu salmon and chum salmon ovarian fluid samples. Frequencies of contamination differed among the 3 salmonid fish species and, thus, factors related to the differences among the species might

contribute to this. The cause of the contamination remained unknown and further study would be required.

Because the nested PCR assay is less laborious, time-consuming and expensive for large numbers of samples (414 samples in this study), the broodstock were screened for *msa* gene DNA by nested PCR assay. Positive samples were then used for real-time PCR assays for *msa* gene mRNA and for *msa* gene DNA. A combination of methods, nested PCR assay and real-time PCR assay for the mRNA, would be advantageous for investigating the mRNA in large numbers of ovarian fluid samples and could be applied to screen large numbers of other tissue samples, including kidney.

In this study detection and quantification of *msa* gene DNA was conducted to examine its correlation to the viability of *Renibacterium salmoninarum* cells in clinically and subclinically infected fish. A good correlation was obtained between the *msa* gene DNA concentration and the culturable cell concentration in kidney tissues of chum salmon fry experimentally challenged by both i.p. injection and bath infection. The R^2 values were 0.992 for i.p. injection and 0.813 for bath infection and were higher than the corresponding R^2 values between the mRNA concentration and the culturable cell concentration (0.924 and 0.502). Furthermore, the prevalence of DNA in the late phase of bath infection (14 and 18 wk after infection) was similar to that of the culturable cells. The results indicate that under certain conditions quantification of *msa* gene DNA is a good indicator of the culturable cell concentration in chum salmon. This conclusion is supported by studies on the correlation between quantification of a gene by quantitative PCR assay and viable cell concentration in bifidobacteria in human feces (Requena et al. 2002, Gueimonde et al. 2004), *Listeria monocytogenes* and *L. innocua* in food (Nogva et al. 2000, Rodríguez-Lazaró et al. 2004), and *Staphylococcus aureus* in food (Hein et al. 2001). Furthermore, prevalence of *Renibacterium salmoninarum* among naturally infected chinook salmon *Oncorhynchus tshawytscha* population was investigated by real-time PCR assay for the bacterial genome DNA and the assay was reportedly valid (Chase et al. 2006, Rhodes et al. 2006). Those studies also support our results.

On the other hand, a discrepancy between the prevalence of *msa* gene DNA and that of culturable *Renibacterium salmoninarum* cells was shown in salmonid kidney tissue in the early phase of bath infection (2 to 10 wk after infection). Furthermore, in salmonid ovarian fluid, the prevalence or detection rate of DNA in the nested PCR assay and real-time PCR assay was higher than the detection rate of the mRNA. These results suggest that a portion of the PCR-positive samples would be false-positive with respect to detection of viable *R. salmoninarum* cells because the real-time PCR assay for mRNA did not show inhibition, therefore, detection rate by the assay would reflect that of viable bacteria. Thus, positive results of the PCR assay should be applied prudently for diagnostic and screening purposes of infected fish to control for BKD by culling or segregating those infected. The presence of gene DNA does not always indicate the presence of viable or culturable bacterial cells because DNA persists in actively killed bacterial cells for a significant period of time (Masters et al. 1994) and also persists in a PCR-detectable form in culture-negative environmental samples (Deere et al. 1996) and clinical samples (Hellyer et al. 1999). Thus, PCR methods to detect bacterial DNA can produce false-positive results through the amplification of target DNA from nonviable bacteria (Josephson et al. 1993, Masters et al. 1994, Sheridan et al. 1998, Keer & Birch 2003). Miriam et al. (1997) reported that PCR for *msa* gene DNA identified much higher numbers in kidney and ovarian fluid samples from commercially reared Atlantic salmon to be positive than did culture and that this may have been due to antibiotic therapy of fish during the previous year.

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