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Development of quantitative RT-PCR targeting *gyr*B mRNA for *Flavobacterium psychrophilum* infecting chum salmon *Oncorhynchus keta*

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サケ冷水病原因菌の*gyr*B mRNAを対象とした定量RT-PCR法の開発

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We developed reverse transcription quantitative real-time PCR (RT-qPCR) targeting *Flavobacterium psychrophilum gyr*B mRNA for rapid detection of live bacteria in chum salmon *Oncorhynchus keta* fry. The mRNA was detected at 6.0×10^{1}–6.0×10^{9} copies/µl with high correlation ($R^2 = 0.9998$). In March 2008, a BCWD outbreak occurred among chum salmon fry in a salmon hatchery in Hokkaido, Japan. The daily mortality peaked in mid–late March and decreased in early April when cumulative mortality reached 2.3%. During the disease course, the culturable cells and *gyr*B mRNA in kidney tissue samples from moribund individuals were identified and quantified by culture and RT-qPCR, respectively. The prevalence and concentration of *gyr*B mRNA was 60% and 4.9×10^{3} copies/mg on March 21, and 100% and 5.6×10^{4} copies/mg on March 26, respectively. No *gyr*B mRNA was detected on March 31, whereas culture prevalence was 95%, 100%, and 40% on March 21, 26, and 31, respectively. These results suggest that the RT-qPCR assay provides rapid quantitative detection of live bacteria in chum salmon fry.

Keywords: bacterial coldwater disease, chum salmon , *Flavobacterium psychrophilum*, *gyr*B mRNA, quantification, RT-qPCR,

Flavobacterium psychrophilum (Bernardet *et al.,* 1996) is the causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (Nematollahi *et al.,* 2003；Barnes and Brown, 2011). This bacterium has been isolated from salmonids, including rainbow trout *Oncorhynchus mykiss* (Lorenzen *et al.,* 1997；Dalsgaard and Madsen, 2000), coho salmon *O. kisutch* (Baliarda *et al.,* 2002；Taylor, 2004), Atlantic salmon *Salmo salar* (Ekman *et al.,* 1999；Cipriano, 2005) and chum salmon *O. keta* (Misaka and Suzuki, 2007). In Japan, BCWD was initially reported in diseased coho salmon in the 1980s (Wakabayashi *et al*, 1991). Since then, rainbow trout (Wakabayashi *et al.,* 1994), masu salmon *O. masou* (Amita *et al.,* 2000) and ayu *Plecoglossus altivelis* (Iida and Mizokami, 1996；Liu *et al.,* 2001) have been affected by BCWD.

In northern Japan, chum salmon is one of the most impor-

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tant fish species for coastal fisheries. In Hokkaido prefecture, located in northern Japan, chum salmon resources have rapidly increased since the 1970s due to an intensive hatchery program (Miyakoshi *et al*., 2013). Around one billion artificially raised chum salmon fry weighing approximately 1–1.5 g (Takahashi, 2015) have been released into the coastal areas of Hokkaido from 145 rivers and 74 net pens in fisheries' ports, and about 40 million adults return to the coastal area annually (Miyakoshi *et al.,* 2016). Recently, we reported a 3.3%–90% prevalence of *F. psychrophilum* in ovarian fluid from returning chum salmon female adults in eight rivers in Hokkaido by culture, and the obtained isolates exhibited virulence in salmonid juveniles including chum salmon (Misaka and Suzuki, 2007). Moreover, chum salmon fry mortality due to BCWD has occurred in several Hokkaido salmon hatcheries, and isolates have been obtained from kidney tissue samples in moribund and dead individuals to date, although the cumulative mortality was less than a few percent in each incidence.

Many diagnostic BCWD methods have been reported to detect *F. psychrophilum,* such as specific antigen detection in tissue samples by immunofluorescence (Madetoja *et al*., 2000；Aoki *et al.,* 2005), specific gene detection by PCR (Urdaci *et al*., 1998；Wiklund *et al.,* 2000), and culturable cells by bacterial culture (Nematollahi *et al.*, 2003；Barnes and Brown, 2011). Immunofluorescence and PCR are rapid and sensitive methods to detect the bacteria, however, these methods detect even though dead bacteria. Culture methods can detect only live bacteria, but are difficult due to the fastidious nature of *F. psychrophilum* (Michel *et al*., 1999；Wiklund *et al*., 2000；Tiirola *et al*., 2002；Nematollahi *et al*., 2003；Barnes and Brown, 2011), and require 48–96 h to produce macroscopic colonies (Holt *et al.,* 1993), resulting in diagnosis delay (del Cerro *et al*., 2002). Thus, rapid and reliable methods are required for detection of live *F. psychrophilum* in fish reared in salmon hatcheries.

Recently, a quantitative real-time PCR (qPCR) assay targeting *F. psychrophilum*-specific genes has been reported (Orieux *et al*., 2011；Marancik and Wiens, 2013；Strepparava *et al.,* 2014). Moreover, to evaluate bacterial growth activities, reverse transcription quantitative real-time PCR (RT-qPCR) targeting mRNA expressed by the *gyr*B gene, encoding the subunit B protein of bacterial DNA gyrase, has been developed for human-related bacteria (Tani *et al*., 2012；Okuno *et al.,* 2015). Izumi and Wakabayashi (2000) described *gyr*B have higher substitution rate of nucleotide and seems to be more appropriate for taxonomic analysis than commonly used 16SrDNA. RT-qPCR assays quantify only live bacterial cells because bacterial mRNA has a short half-life, measured in minutes, and is, therefore, a good indicator of cell viability (Arraiano *et al*., 1988；Belasco, 1993；Alifano *et al.,* 1994；Sheridan *et al.,* 1998). Additionally, RT-qPCR takes only several hours to perform, providing rapid diagnosis.

The purpose of this study is to develop RT-qPCR targeting *gyr*B mRNA of *F. psychrophilum* to detect live bacteria rapidly and to evaluate the usefulness of this method in chum salmon fry naturally infected with the bacterium.

MATERIALS AND METHODS

Bacterial strains and growth conditions Twelve *Flavobacterium psychrophilum* strains and 13 other bacterial strains were used to verify the specificity of *gyr*B qPCR and *gyr*B mRNA RT-qPCR assay. *F. psychrophilum* strains were previously isolated from salmonids including chum salmon, masu salmon, coho salmon, rainbow trout, and ayu, and were cultured using modified *Cytophaga* broth (CBm；Wakabayashi and Egusa, 1974). The species, origin, and culture conditions of the other bacteria are summarized in Table 1 .

Batch culture *F. psychrophilum* type strain NCIMB 1947^{T} was cultured in CBm while shaking for 72 h at 15 °C. At 0, 9, 24, 33, 48, 53, and 72 h post inoculation, an aliquot of the bacterial culture was sampled. One ml of each aliquot of the bacterial culture was used for both qPCR of the *gyr*B gene and the RT-qPCR assay for *gyr*B mRNA. A 100 µL sample of each bacterial culture aliquot was also serially diluted 10-fold in CBm, inoculated onto CAm (CBm with 1.5% agar), and incubated for 4–5 days at 15 °C to examine culturable cell numbers, which were expressed as colony forming units (CFU). qPCR and RT-qPCR were conducted as described below.

Cloning of *gyr*B gene To clone a part of *F. psychrophilum* type strain NCIMB 1947^{T} *gyr*B gene, PCR was performed using primers PSY-G1F and PSY-G1R to amplify 1,017 base pair product (Izumi and Wakabayashi, 2000). PCR amplification was performed using a GeneAmp® 2400 PCR thermal cycler (Applied Biosystems) in a 25 µL reaction mixture containing 1 µL of DNA template (100 µg/mL), 0.2 µM of each primer, and 12.5 µL of AmpliTaq Gold® PCR Master Mix (Applied Biosystems). Cycling conditions were

Table 1 Strains, hosts, and culture medium of *Flavobacterium psychrophilum* and other bacteria used to verify the specificity of reverse transcription quantitative real-time PCR assay

Species	Strain	Host fish	tissue	Isolate year	Culture medium	Remarks
Flavobacterium psychrophilum	NCIMB1947^T	coho salmon		Unknown	modified *Cytophaga* [a]	
F. psychrophilum	FPC814	rainbow trout		1991	modified *Cytophaga*	
F. psychrophilum	FPC840	ayu		1987	modified *Cytophaga*	
F. psychrophilum	Fa-A	ayu	kidney	2002	modified *Cytophaga*	Misaka and Suzuki (2007)
F. psychrophilum	Fp-B	coho salmon	kidney	2002	modified *Cytophaga*	Misaka and Suzuki (2007)
F. psychrophilum	Fp-C	masu salmon	kidney	2002	modified *Cytophaga*	Misaka and Suzuki (2007)
F. psychrophilum	Fp-D	rainbow trout	kidney	2002	modified *Cytophaga*	Misaka and Suzuki (2007)
F. psychrophilum	Fp-K	chum salmon	Ovarian fluid	2004	modified *Cytophaga*	Misaka and Suzuki (2007)
F. psychrophilum	Fp-O	chum salmon	Ovarian fluid	2004	modified *Cytophaga*	Misaka and Suzuki (2007)
F. psychrophilum	Fp-T	chum salmon	kidney	2005	modified *Cytophaga*	Misaka and Suzuki (2007)
F. psychrophilum	Fp-W	masu salmon	kidney	2005	modified *Cytophaga*	Misaka and Suzuki (2007)
F. psychrophilum	Fp-Z	chum salmon	kidney	2005	modified *Cytophaga*	Misaka and Suzuki (2007)
F. branchiophilum	ATCC35035				modified *Cytophaga*	
F. limicola	NBRC103156^T				tryptic soy broth	
F. granuli	NBRC102009^T				R2A [b]	
F. columnare	Fc-1	chum salmon	caudal pedancle	2005	modified *Cytophaga*	
Pseudomonas flavescens	NBRC103044^T				702 medium [c]	
P. fluorescens	NBRC101042				702 medium	
Aeromonas salmonicida	NCIMB1102				tryptic soy broth	
Vibrio anguillarum	NCIMB828				tryptic soy broth	
Bacillus subtilis	ATCC6633				tryptic soy broth	
B. cereus	ATCC1178				tryptic soy broth	
Micrococcus luteus	ATCC9341				tryptic soy broth	
Chryseobacterium daecheongense	NBRC102008^T				R2A	
Renibacterium salmoninarum	Rs-3	chum salmon	kidney	2004	SKDM [d]	Suzuki and Sakai (2007)

a: Wakabayashi &Egusa (1974) b: Reasoner & Geldreich (1985) c:Takehara et al (2008) d:Austin et al. (1983)

an initial denaturation at 95°C for 10 min；followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min；and a final extension at 72°C for 7 min. An aliquot of PCR product (15 μL) was electrophoresed on a 0.8% agarose gel containing 50 μg/mL ethidium bromide. The target band (1,017 base pairs) was purified using a Suprec-01™ filter cartridge (Takara Bio Inc.), and then ligated into pGEM®-T Easy Vectors (Promega). The recombinant plasmid pGEM-T/*gyr*B was transformed into *Escherichia coli* JM109 (Takara Bio Inc.).

In vitro transcription of standards The recombinant plasmid was extracted from the transformed *E. coli* using a Flexi Prep Kit (Amersham Bioscience), linearized with the restriction enzyme *Sal*I, and purified using Suprec-01™ (Takara Bio Inc.) filtration cartridges. The single strand RNA transcripts were produced using a MEGAscript® T7 Transcription Kit (Ambion) and electrophoresed on MOPS/formaldehyde gels to confirm the presence of single bands of the target size. The transcripts were purified using a MEGAscript® T7 Kit, and the absence of plasmid DNA in the aliquot was confirmed by PCR targeting the *gyr*B gene. The RNA yield and purity of the transcripts were determined spectrophotometrically by measuring the absorbance ratio at 260/280 nm. The transcript concentration was calculated by the molecular weight and Avogádro number. Serial 10-fold dilutions of the transcripts at 6.0×10^1–6.0×10^9 copies/μL in NANOpure water (Barnstead/Thermolyne Corp.) were used as standards for the RT-qPCR assay described below.

cDNA synthesis cDNA synthesis of RNA standard samples and unknown samples was conducted using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Evaluation of reverse transcription efficiency An RNA standard serially diluted from 6.0×10^1–6.0×10^9 copies/μL was reverse transcribed to cDNA and as used as a qPCR template determine the efficiency of reverse transcription. Samples were diluted 2-fold for cDNA synthesis. The cDNA samples were stored at −20°C.

RNA extraction Total RNA from bacterial culture samples and kidney tissue samples from chum salmon fry stored in RNA*later*™ solution (Ambion) were extracted using an RNAqueous®-4PCR Kit (Ambion). Briefly, 1 mL of bacterial culture sample centrifuged at $8{,}000 \times g$ for 5 min at 4°C and the kidney tissue samples collected aseptically (10-20 mg per fish) were mixed with 100 μL of RNA*later*™ solution and stored at 4°C for 24 h. Then, the bacterial culture and kidney samples were centrifuged at $8{,}000 \times g$ for 5 min at 4°C, and the supernatants were discarded. The samples

were stored at −80°C until analysis. No genomic DNA remained because following RNA extraction, DNA in the samples was digested using a Turbo DNA-free™ Kit (Ambion). Thus, the samples were diluted 1.2-fold.

DNA extraction DNA was extracted from bacterial culture samples and kidney tissue samples (10–20 mg per fish) using a Sepa Gene kit (Eidia Co. Ltd). The extracted DNA was dissolved in 1 µL of NANOpure water per 1 mg kidney tissue or 10 µL of bacterial culture. The DNA yield and purity were determined spectrophotometrically by measuring the absorbance ratio at 260/280 nm.

RT-qPCR assay for *gyr*B mRNA To quantify cDNA generated by reverse transcription of RNA, RT-qPCR was conducted with a TaqMan probe using a 7500 Real-Time PCR System (Applied Biosystems). The primers and probe were designed using Primer Express 2.0 software (Applied Biosystems) based on the nucleotide sequence of the *gyr*B gene paralog *par*E of *F. psychrophilum* strain FPC828 (GenBank accession number AB012859). The sequences of forward primer PSY-945GF, reverse primer PSY-1015GR, and TaqMan probe PSY-966GP were 5'-CGGCTTCGGGTTCTATCACA-3', 5'-AGGCTTACCACGCAAGCTAAAC-3', and 5'-AATCTCGTGATGTAAATAC-3', respectively. The TaqMan probe consisted of an oligonucleotide with a 5' 6-carboxyfluorescein (FAM) reporter fluorescent dye and a 3' nonfluorescent quencher plus minor groove binder. The 50 µL reaction mixture contained 25 µL of TaqMan® Universal PCR Master Mix with no UNG (Applied Biosystems), 0.9 µM of each primer, 0.25 µM of TaqMan probe, and 5 µL of cDNA (10 ng/µL). The reactions were performed in duplicate for each sample. The amplification program was 95°C for 10 min followed by 45 cycles at 95°C for 15 s and 60°C for 1 min.

Specificity determination of primers and probe To determine the specificity of the primers and probe, DNA extracted from the stationary phase of the pure bacteria cultures, including 12 strains of *F. psychrophilum* and 13 strains of other bacteria (Table 1) were quantified by qPCR. The DNA concentration in the samples was adjusted to approximately 100 µg/mL.

Evaluation of the extraction efficiency of *gyr*B mRNA in kidney tissue samples The efficiency of the *gyr*B mRNA extraction from kidney tissue samples was evaluated by RT-qPCR. Kidney tissue samples (approximately 10 mg per fish) were collected from apparently healthy chum salmon fry weighing around 1 g, mixed with 100 µL of RNA*later*™, and stored at 4°C for 24 h. Kidney tissue samples from 10 individuals were pooled, and 20 mg of the pooled samples were spiked with 10 µL of RNA standard samples at concentrations of 6.0×10^3, 6.0×10^5, 6.0×10^7, and 6.0×10^9 *gyr*B mRNA copies/µL and then homogenized. NANOpure water was used as a non-spiked control. Total RNA was extracted from the spiked samples.

The RNA concentration in each sample was determined by RT-qPCR assay, and the extraction efficiency was calculated.

Fish from naturally occurring BCWD outbreaks In early March 2008, a BCWD outbreak occurred in around 750,000 chum salmon fry, each weighing approximately 1 g, reared in a pond at a salmon hatchery in Hokkaido, Japan. Number of dead fry was counted everyday and daily mortality was recorded from March 5 to April 5. Water temperature was recorded from March 5 to April 5. In early March, a low percentage of individuals exhibited disease symptoms including a slightly eroded caudal fin and anemia. From these observations, we concluded a possible BCWD outbreak since this hatchery had a BCWD outbreak in March and April of the previous year (2007). In this previous outbreak, moribund and dead chum salmon fry reared in the facility displayed symptoms typical of BCWD including a severely eroded caudal fin, spleen hypertrophy, and anemia. *F. psychrophilum* was isolated from most of the kidney tissue samples of the dead individuals.

Kidney tissue samples (10–20 mg per fish) from 25 individuals of the 2008 outbreak were collected on March 21, 26, and 31, respectively. Cold CBm was used to homogenize 20 of these samples (100 µL per sample), and they were cultured using CAm at 15°C for 5 days. Five of these samples were used for RT-qPCR described above.

Correlation analyses Linear regression and square regression coefficient (R^2) were analyzed using Microsoft Excel 2007 (Microsoft).

RESULTS

Specificity of primers and probe The specificity of the primers and the TaqMan probe was confirmed. The concentration of *gyr*B in culture samples from 12 strains of *Flavobacterium psychrophilum* was $1.2–8.1 \times 10^7$ copies/µL, whereas no amplification products were detected in the 13 strains of other bacteria except for *F. branchiophilum,*

Table 2 Threshold PCR cycle number (C^T) and quantification of *gyr*B DNA of *Flavobacterium psychrophilum* and other bacteria by quantitative real-time PCR assay

Species	C^T	Copies μl^{-1}
Flavobacterium psychrophilum	17.60	1.2×10^7
F. psychrophilum	15.04	6.6×10^7
F. psychrophilum	15.18	6.0×10^7
F. psychrophilum	17.46	1.3×10^7
F. psychrophilum	15.20	6.0×10^7
F. psychrophilum	14.74	8.1×10^7
F. psychrophilum	17.54	1.3×10^7
F. psychrophilum	16.48	2.6×10^7
F. psychrophilum	16.70	2.2×10^7
F. psychrophilum	15.26	5.7×10^7
F. psychrophilum	16.02	3.5×10^7
F. psychrophilum	17.04	1.8×10^7
F. branchiophilum	35.46	7.8×10^1
F. limicola		N.D.[a]
F. granuli		N.D.
F. columnare		N.D.
Pseudomonas flavescens		N.D.
P. fluorescens		N.D.
Aeromonas salmonicida		N.D.
Vibrio anguillarum		N.D.
Bacillus subtilis		N.D.
B. cereus		N.D.
Micrococcus luteus		N.D.
Chryseobacterium daecheongense		N.D.
Renibacterium salmoninarum		N.D.

a:Not detected

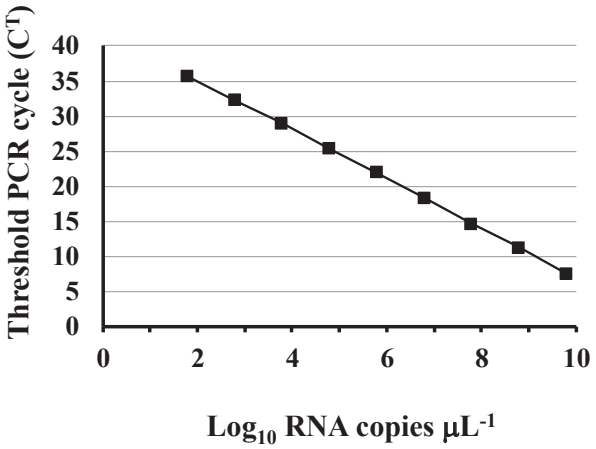

Fig. 1 Standard curve of threshold PCR cycles (C^T) versus log concentration of *gyr*B gene cDNA by reverse transcription of mRNA in *Flavobacterium psychrophilum*

which yielded 7.8×10^1 copies/μL by the assay (Table 2).

Reliability and detection limit of RT-qPCR assay for *gyr*B mRNA The threshold PCR cycle number (C^T) values plotted against the standard samples (log concentration of *gyr*B mRNA) gave a regression line from 6.0×10^1–6.0×10^9 copies/μL (Fig. 1). The R^2 value was 0.9998, demonstrating a high correlation. The slope of the reaction was −3.51, and the amplification efficiency was 0.927. The lower detection limit in the reaction was 6.0×10^1 copies/μL, corresponding to 1.4×10^2 copies/mg of kidney tissue samples.

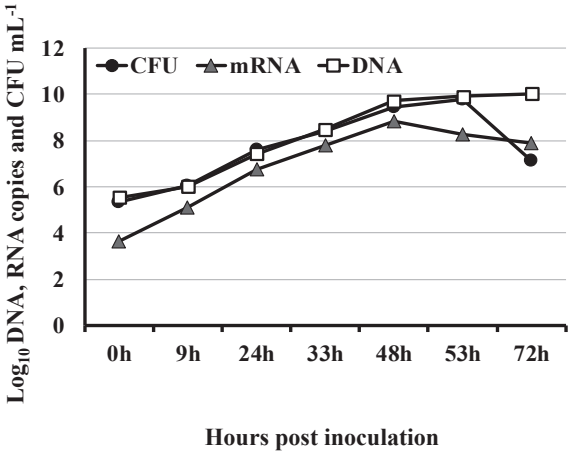

Fig. 2 Transition in concentrations of DNA, mRNA and culturable cell number (CFU) of *Flavobacterium psychrophilum* during batch culture using *Cytophaga* broth at 15°C

Evaluation of reverse transcription and extraction efficiency of *gyr*B mRNA in kidney tissue samples The efficiency of reverse transcription from serially diluted RNA samples at 6.0×10^3–6.0×10^9 copies/μL to cDNA was almost 100% (range, 91.3%–126.6%). The extraction efficiency (ratio of the concentration of RNA standard samples to the concentration of spiked RNA standard samples in kidney tissue) was approximately 40% (Table 3) because the samples were diluted 5-fold in extracting RNA, 1.2-fold in DNase treatment and 2-fold in reverse transcription (total 12-fold).

Batch culture The culturable number of bacteria was 2.2×10^5 CFU/mL at 0 h post inoculation. It increased to 5.8 x 10^9 CFU/mL on 53 h, and decreased to 1.4 x 10^7 CFU/mL on 72 h post inoculation (Fig. 2). The *gyr*B mRNA concentration at 0 h post inoculation was 4.2×10^3 copies/mL. It increased to 6.5 x 10^8 copies/mL on 48 h, and decreased to 7.8 x 10^7 copies/mL on 72 h post inoculation. The *gyr*B DNA concentration was 3.5×10^5 copies/mL at 0 h post inoculation, continued to increase during culture and reached to 1.1 x 10^{10} copies/mL on 72 h post inoculation. Thus, *gyr*B mRNA concentration to culturable cell count (CFU) or DNA concentration was well correlated in the log phase of the culture. The ratio of mRNA concentration to culturable cell concentration was approximately 0.1–0.2 in log phase and 0.03–0.6 in stationary phase.

Table 3　Extraction efficiency of RNA in spiked kidney tissues by reverse transcription real-time PCR assay

Standard samples	Spiked samples		Extraction efficiency
RNA copies μl^{-1} (A)	Threshold PCR cycle number (C^T)	RNA copies μl^{-1} (B)*	(B)/(A) (%)
6.0×10^9	12.19	1.9×10^8	37.4
6.0×10^7	19.15	1.9×10^6	37.4
6.0×10^5	25.74	2.4×10^4	48.0
6.0×10^3	32.89	2.1×10^2	39.6
None (control)	37.04	1.4×10^1	-

* The samples were diluted 12-fold (diluted 5-fold in extracting RNA, 1.2-fold Dnase treatment and 2-fold in reverse transcription)

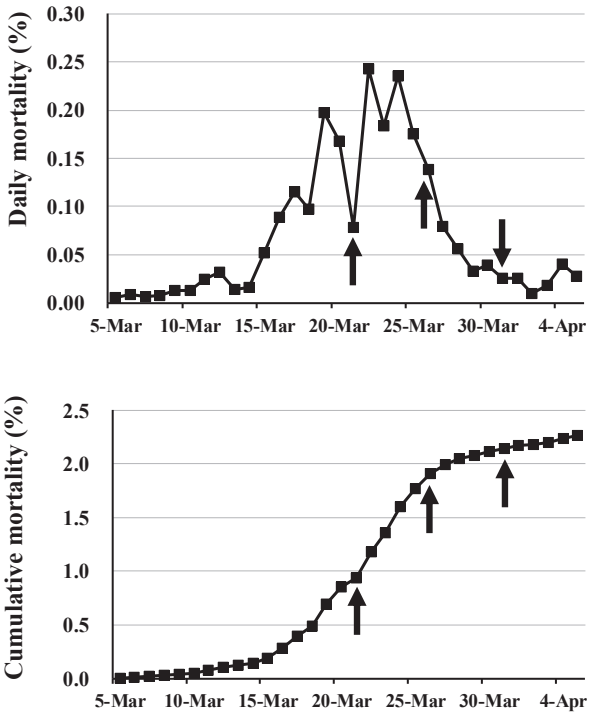

Fig. 3　Transition in daily mortality rate (a) and cumulative mortality rate (b) of chum salmon fry due to BCWD in a hatchery. Arrows show sampling date.

Naturally infected chum salmon fry　In March 2008, a low percentage of chum salmon fry (approximately 10,000–20,000 individuals) reared in one pond in a hatchery exhibited disease symptoms including a slightly eroded caudal fin typical of BCWD. Daily mortality increased to 0.05% on March 15 and peaked at 0.24% from March 22 to 24, finally decreasing to 0.03% in late March (Fig. 3a). The cumulative mortality reached 2.3% on April 5 (Fig. 3b). The water temperature in this pond was recorded everyday, and gradually increased from 7.5 °C on March 5 to 9.2 °C on April 5. The prevalence by culture (number of positive samples/number of samples tested) was 95% on March 21, increasing to 100% on March 26 and decreasing to 40% on March 31 (Table 4). On March 21, the prevalence and concentration of *gyr*B mRNA, as determined by RT-qPCR, were 60% and 4.9 × 10^3 copies/mg kidney tissue, respectively, 100 % and 5.6 × 10^4 copies/mg on March 26. On March 31, *gyr*B mRNA was not detected in any samples.

DISCUSSION

Specificity of RT-qPCR　This study revealed that the specificity of the RT-qPCR assay targeting *gyr*B mRNA was sufficiently high because all strains of *Flavobacterium psychrophilum* were detected and quantified at the same level, whereas no amplification was detected in the other bacteria used except for *F. branchiophilum*, showing a weak cross-reaction. *F. branchiophilum* is the causative agent of bacterial gill disease in freshwater fishes (Wakabayashi *et al*., 1989；Bullock, 1990) and has been isolated from salmonids (Heo *et al.,* 1990；Turnbull, 1993). However, the bacterium has only been detected in external organs including gill tissue and on the skin's surface and has not been reported in internal organs (kidney tissue and others) to date (Wakabayashi, 2004). Strepparava *et al*. (2014) reported a weak cross-reaction with the highest *F. branchiophilum* pure DNA concentrations (10^6 cells per reaction and 50 copies detected) in a qPCR targeting *F. psychrophilum rpo*C, encoding RNA polymerase β' subunit, and considered the results as negative. This finding is consistent with our results. Thus, the weak cross-reaction with *F. branchiophilum* observed in our study could probably be negligible for quantitative detection of *F. psychrophilum* in chum salmon fry. Our report is the first on RT-qPCR targeting *F. psychrophilum gyr*B mRNA for rapid detection of live bacteria. The specificity of designed primers and probe used in this study targeting specific region of *gyr*B gene of *F. psychrophilum* is sufficiently high because all *F. psychrophilum* strains used in this study were detected same level in RT-qPCR as described above. However, the *gyr*B gene sequence corresponding to PSY-945GF and PSY-1015GR in

Table 4 Detection rate and average concentration of *gyr*B mRNA of *Flavobacterium psychrophilum* in chum salmon fry kidney tissue samples of during naturally occurring outbreak of bacterial coldwater disease Numbers in parethesis indicate minimum and maximum value

Sampling date	Culture	RT-qPCR	
	Prevalence (%)	Prevalence (%)	Concentration (copies mg-1 of kidney tissue)
Mar 21	95	60	4.9×10^3 (2.2×10^3 - 1.6×10^4)
Mar 26	100	100	5.6×10^4 (1.1×10^3 - 1.2×10^5)
Mar 31	40	ND*	ND

* Not detected

the strain OKA9805 isolated from ayu (Izumi and Wakabayashi, 2000) has one mutation in each primer region (accession number AB034737). Izumi *et al*. (2003) suggested *F. psychrophilum* strains isolated from ayu have unique genotype compared to strains isolated from other species by PCR-RFLP analysis. So developed method in this study might not be suitable for detection of *F. psychrophilum* in ayu.

Effectiveness of RT-qPCR Bacterial mRNA has a short half-life, measured in minutes, and could be a good indicator of cell viability (Arraiano *et al*., 1988；Belasco, 1993；Alifano *et al*., 1994；Coutard *et al.,* 2005；Elliot, 2012). The RT-qPCR assay developed in this study could be a rapid and reliable method to quantify specific mRNA of *F. psychrophilum* and could quantify *F. psychrophilum*-specific mRNA despite the presence of other bacteria, thereby allowing the detection of live, proliferative bacteria in chum salmon fry reared in salmon hatcheries. In *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease, RT-PCR was used to detect viable cells (Cook and Lynch, 1999), and RT-qPCR was verified to quantify the pathogen viability (Suzuki and Sakai, 2007).

Bacterial culture is a renowned method to quantify live proliferative bacteria because it can be conducted inexpensively and has a higher sensitivity. However, culture takes a relatively long time to produce macroscopic colonies, subculture and identification of bacteria such as PCR (usually 5–8 days). Additionally, other fast-growing bacteria could interfere with colony detection (Kumagai *et al*., 2004).

This study showed a relatively low sensitivity of the RT-qPCR assay compared to that of culture. Although in batch culture the ratio of the concentration of *gyr*B mRNA to that of the culturable cell concentration was well correlated in log phase at 9–48 h post inoculation, the ratio of mRNA concentration to culturable cell (or DNA) concentration was approximately 0.1–0.2. This suggests a low mRNA concentration in the kidney tissue samples of moribund fish in BCWD outbreaks as well as in batch culture and a somewhat lower sensitivity of the RT-qPCR assay for field fish samples compared to that of culture methods. Perhaps the extraction efficiency of RNA being approximately 40%, which was estimated by a spiking test, may be attributed to these results. However, irrespective of this disadvantage, *gyr*B mRNA could be an indicator of *F. psychrophilum* growth activity. Additionally, the slope of reaction between the threshold PCR cycle number and log concentration in RNA standard samples or spiked RNA standard samples in kidney tissue were almost same, -3.51 and -3.43, respectively (data not shown). This result indicates the effectiveness of RT-qPCR. The *gyr*B gene encodes subunit B of bacterial DNA gyrase, playing an essential role in DNA replication in all bacterial species (Roberts and Shapiro, 1997). Furthermore, *gyr*B gene is a housekeeping gene, and a single copy exists in most bacterial genomes. Wu *et al*. (2015) reported complete genome sequence of *F. psychrophilum* strain ATCC 49418^T (=NCIMB1947^T), and this strain has single copy of *gyr*B gene (accession number CP007207). Thus, expression of *gyr*B gene can be used as an indicator of bacterial growth (Tani *et al*., 2012).

Relationship among mRNA, DNA and CFU The expression levels in *F. psychrophilum* obtained in this study were generally lower than other human-related bacterial spe-

cies including *Escherichia coli* (Tani *et al*., 2012) and *Legionella pneumophila* (Okuno *et al*., 2015). *Gyr*B expression in *F. psychrophilum* was upregulated at 8°C compared with 20°C (Hesami *et al*., 2011). Batch culture was conducted at 15°C in this study, however, water temperature in hatcheries rearing chum salmon fry in Hokkaido below 10°C in many cases. BCWD typically occurs at water temperature below 16°C and most prevalent and serious at 10°C and below (Starliper, 2011). Also, we conducted batch culture experiment up to 72 h post inoculation, the mRNA expression peaked on 48 h. Suzuki and Sakai (2007) has conducted batch culture experiment using *Renibacterium salmoninarum* up to 34 days post inoculation, and they confirmed the correlation among CFU, concentration of DNA and mRNA of bacterium through the experiment nevertheless the mRNA expression peaked on 14 days. Thus, we should conduct batch culture experiment in different temperature using various strains of *F. psychrophilum* for longer period to confirm the reliability of this study.

In this study in a salmon hatchery with a history of BCWD, the disease reoccurred in chum salmon fry reared in the facility. The fish showed disease signs typical of BCWD and mortality due to the disease. The pathogen among the rearing fry were detected and quantified by RT-qPCR for *gyr*B mRNA, which could reflect the mortality profile of the disease. The pathogen increased as the disease progressed from March 21 to March 26. No *gyr*B mRNA was detected on March 31, when the daily mortality decreased to low levels. Culture prevalence also coincided well with the disease course, and the sensitivity of culture method was higher than that of RT-qPCR. These results suggest that the RT-qPCR assay allowed for the rapid and reliable detection of live and proliferative *F. psychrophilum* quantitatively in chum salmon fry in salmon hatcheries. The cause of the discrepancy between prevalence by culture and that by RT-qPCR on March 31 remains unclear. The difference of sample number (RT-qPCR; n = 5, culture ; n = 20) may attribute this result. *F. psychrophilum* has been reported as a subclinical infection in some salmonid populations (Dalsgaard and Madsen, 2000 ; Wiklund *et al.,* 2000 ; Madetoja *et al*., 2000). A subclinical infection or pathogen carriers in chum salmon fry and a somewhat higher sensitivity of the culture method compared with that of the RT-qPCR method might have influenced the results.

In this study, disease might already a bit progressed in chum salmon fry sampled in a hatchery on March 21, so artificial infection experiment and sampling of diseased fish during course of the disease must be conducted to confirm the usefulness RT-qPCR method. Many artificial infection of *F. psychrophilum* have been conducted by injection experiment (Madsen and Dalsgaard, 1999 ; Garcia *et al*., 2000). These methods bypass the non-specific defense mechanism located in the skin, so do not appropriate to evaluate the concentration of pathogen in internal organs. Immersion infection experiment is appropriate method, however, establishment of infection could difficult without some treatment (Madsen and Dalsgaard, 1999 ; Madetoja *et al*., 2000). Although we failed to several immersion infection experiments using chum salmon fry, immersion infection experiment is needed to apply the RT-qPCR method to rapid diagnosis of BCWD. In analysis of RT-qPCR, PCR and culture using chum salmon fry, another fish has used in each method. Using same organ from one individual is favorable to compare the results among these methods, however, chum salmon fry in hatchery is small described above and is difficult to collect kidney tissue samples for three methods from one individual. Further experiment is needed using more big fish to compare the usefulness of these methods.

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