Short communication

PCR- Based Detection and Quantification of *Renibacterium salmoninarum* in Ovarian Fluid of Returning Chum Salmon *Oncorhynchus keta* and Masu Salmon *O. masou* in Hokkaido, Japan

Kunio Suzuki*, Naoyuki Misaka, Shinya Mizuno and Yoshitaka Sasaki

Salmon and Freshwater Fisheries Research Institute,
Hokkaido Research Organization,
Hokkaido 061-1433, Japan

(Received February 6, 2017)

ABSTRACT—*Renibacterium salmoninarum* was detected in ovarian fluid of returning salmonids using nested PCR; chum salmon from seven rivers (925 samples) and masu salmon from three rivers (309 samples) in Hokkaido in 2015 and 2016. Real-time PCR was also performed for the samples in 2016. In chum salmon prevalence of *R. salmoninarum* genome was 0%–90% in 2015 and 12%–98% in 2016. In masu salmon the prevalence was 0% in 2015 and 50%–84% in 2016. In 2016, the prevalence had risen in the samples in eight of ten rivers. The numbers of *R. salmoninarum* genome were estimated to be 1.0×10^1 – 8.0×10^2 copies/ μ L of ovarian fluid.

Key words: *Renibacterium salmoninarum*, prevalence, *Oncorhynchus keta*, *Oncorhynchus masou*, ovarian fluid

The detection of fish pathogens in adult salmon returning to rivers is critical for sustainable salmon enhancement programs. Previous researchers have used ovarian fluid samples from adult salmon returning to rivers in Hokkaido to detect viruses harmful to fish (Yoshimizu *et al.*, 1988; Kasai *et al.*, 2003) as well as *Aeromonas salmonicida* bacterium, the causative agent of furunculosis (Nomura and Kimura, 1981; Nomura *et al.*, 1991; Nomura *et al.*, 1992). These studies have also reported on prevalence of the pathogens in the samples. However, an estimation of infection intensity (quantity) of the pathogens in the samples, using

quantitative diagnostic methods, is also needed for the evaluation of infection levels (prevalence and intensity) for control of transmission and progression of bacterial kidney disease (BKD) (Pascho *et al.*, 1991; Maule *et al.*, 1996; Faisal and Eissa, 2009).

BKD is a slow and progressive disease caused by *Renibacterium salmoninarum*, which threatens wild and farm-raised salmonid stocks throughout the world (Evenden *et al.*, 1993). Suzuki and Sakai (2007) reported *R. salmoninarum* infection levels in chum salmon returning to eight rivers in Hokkaido, in 2005, as detected in ovarian fluid samples using nested PCR (nPCR) and quantitative real-time PCR (qPCR). Since this study, infection levels of *R. salmoninarum* in Hokkaido salmonids have not been further examined and thus remains unclear. Therefore, we examined prevalence of the bacterium in ovarian fluid of adult chum and masu salmon returning to seven and three rivers in Hokkaido, respectively, sampled in 2015 and 2016, using nPCR. In addition, qPCR was conducted to examine infection intensity of *R. salmoninarum* in the samples obtained in 2016.

Materials and Methods

Collection of ovarian fluid

Adult chum salmon female were collected from seven rivers (River A–G, Table 1) and then reared at seven separate hatcheries where BKD had never been reported (Sites A–G, Table 1). Individual ovarian fluid samples (approximately 0.5 mL per fish) were taken from 60 randomly selected fish at each of the seven hatcheries using a sterile tip (Yoshimizu *et al.*, 1985). Adult masu salmon females were collected from three other rivers in Hokkaido (Site H–J, Table 1), and ovarian fluid samples were likewise obtained.

DNA extraction

DNAs were extracted from 50 μ L of ovarian fluid samples using SepaGene Kit (Eidia). Extracted DNAs were dissolved in 50 μ L of ultrapure water; hence, one micro liter of the solution contained DNA from 1 μ L ovarian fluid.

nPCR assay for major soluble antigen (*msa*) gene

An nPCR assay was performed according to Pascho *et al.* (1998), with slight modifications. Briefly, two different pairs of primers were used for first-round PCR and second-round PCR (P3 and M21, and P4 and M38, respectively). The 25- μ L reaction mixture contained 12.5 μ L of AmpliTaq Gold 360 Master Mix (Applied Biosystems), 0.5 μ M of each primer, and 2 μ L of DNA solution. In the second-round PCR, 1 μ L of first-round PCR product was used as the template DNA. The amplification program of the first-round PCR was one cycle of 95°C for 10 min, and 30 cycles of 94°C for

* Corresponding author
E-mail: suzuki-kunio@hro.or.jp

Table 1. *Renibacterium salmoninarum* detected in ovarian fluid samples from returning chum and masu salmon female adults in 2015 and 2016 by the nested PCR and by the quantitative real-time PCR assay for *msa* gene of the pathogen

Species	Sampling			Nested PCR	Quantitative real-time PCR
	Site	Date	Number	No. of positive/No. of sample tested (prevalence[%])	Genome copies/ μ L of ovarian fluid
Chum	A	Oct 2015	60	54/60 (90%)	Not done
		Sep 2016	61	22/60 (37%)	1.0×10^1 – 2.2×10^2
		Oct 2016	60	59/60 (98%)	1.2×10^1 – 3.5×10^2
		Nov 2016	60	15/60 (25%)	1.0×10^1 – 3.9×10^1
	B	Oct 2015	24	1/24 (4%)	Not done
		Oct 2016	60	14/60 (23%)	1.0×10^1 – 1.2×10^2
	C	Oct 2015	60	0/60 (0%)	Not done
		Oct 2016	60	27/60 (45%)	1.0×10^1 – 2.0×10^2
	D	Oct 2015	60	25/60 (42%)	Not done
		Oct 2016	60	7/60 (12%)	2.3×10^1
	E	Oct 2015	60	0/60 (0%)	Not done
		Oct 2016	60	34/60 (57%)	1.4×10^1 – 3.5×10^1
	F	Oct 2015	60	48/60 (80%)	Not done
		Oct 2016	60	44/60 (73%)	1.2×10^1 – 2.5×10^2
	G	Nov 2015	60	18/60 (30%)	Not done
		Nov 2016	60	36/60 (60%)	1.5×10^1 – 8.0×10^2
Masu	H	Sep 2015	60	0/60 (0%)	Not done
		Sep 2016	61	51/61 (84%)	1.4×10^1 – 1.6×10^2
	I	Sep 2015	60	0/60 (0%)	Not done
		Sep 2016	8	4/8 (50%)	6.5×10^1 – 7.4×10^1
	J	Sep 2015	60	0/60 (0%)	Not done
		Sep 2016	60	48/60 (80%)	1.1×10^1 – 2.1×10^2

30 s, 60°C for 1 min and 72°C for 1 min. The amplification program of the second-round PCR was the same as that of the first-round PCR, but employed a different pair of primers. The detection limit of the assay was 1–9 genome copies/ μ L DNA solution in a reaction, and it was more sensitive than the qPCR assay, described below. The nPCR assay produces a 320 bp amplification product.

qPCR for *msa* gene

A qPCR assay was conducted as previously described by Suzuki and Sakai (2007), with slight modifications. Briefly, the 25- μ L reaction mixture contained 12.5 μ L of TaqMan Universal Master Mix II with UNG (Applied Biosystems), 0.9 μ M of each primer (1177F and 1313R), 0.4 μ M TaqMan probe (1231T) and 2.5 μ L of DNA solution. The amplification condition was one cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were performed in an Applied Biosystems QuantStudio 6 Flex qPCR System.

DNA extracted from the cultured *R. salmoninarum* strain ATCC 33209 was 10-fold serially diluted with ultra-pure water at 1.0×10^1 – 1.0×10^7 genome copies/ μ L and used as a standard sample. The detection limit of the qPCR assay was 10 genome copies/ μ L DNA solution in a reaction. The qPCR assay was performed for a DNA sample positive for *R. salmoninarum* genome in the nPCR, as that was more sensitive than the qPCR assay

used in this study.

Results and Discussion

In total, 925 ovarian fluid samples were obtained from chum salmon collected in 2015 and 2016 (Table 1). On the results of bacterial genome detection in the samples by nPCR (Fig. 1), prevalence of the bacterium was estimated to be 0%–90% in the samples in 2015, and 12%–98% in 2016 samples, representing salmon derived from seven rivers (sites) in Hokkaido. Prevalence in ovarian fluid obtained from six sites (but not Site D) showed similar or higher values for 2016 than occurred in 2015 samples. We are not able to speculate on the cause of this increase in 2016. Concentrations of bacterial genome estimated by qPCR, which indicates infection intensity of the bacterium in the fish, were 1.0×10^1 – 8.0×10^2 copies/ μ L of ovarian fluid, inclusive of seven sites (Table 1). These results demonstrate that infection levels (prevalence and intensity) greatly differed among the seven sites, and the three sites (Sites A, F, G) in particular showed higher infection levels than the other sites.

For masu salmon, a total of 309 ovarian fluid samples were examined by nPCR. The prevalence of the bacteria in samples from the three sites investigated was 0% in 2015, but 50%–84% in 2016 (Table 1). Thus, prevalence had also increased in the masu salmon in

Fig. 1. Agarose gel electrophoresis of nested PCR (nPCR) products from the DNA extract of ovarian fluid sample of chum salmon collected in Hokkaido in 2016. Lane 1, DNA marker; Lane 2, positive control; Lane 3, negative control; Lane 4–10, DNA extract of chum salmon ovarian fluid samples collected at Site A in Oct 2016.

2016. The concentrations of the bacterial genome estimated by qPCR were 1.1×10^1 – 2.1×10^2 copies/ μ L of ovarian fluid.

This study showed that infection levels (prevalence and intensity) of *R. salmoninarum* in the ovarian fluid of both salmon species probably increased in 2016 as compared with those detected in 2005 and 2015. Suzuki and Sakai (2007) reported 0%–30% prevalence of the bacterium in ovarian fluid of returning adult chum salmon in eight rivers in Hokkaido, as detected by nPCR assay, and the concentration of genome was 1.1×10^1 – 1.2×10^2 copies/ μ L of ovarian fluid by qPCR assay. Similar concentrations of the genome were found in this study. However, it is difficult to compare the quantitative findings of this study with the results of Suzuki and Sakai (2007): whereas we used genomic DNA of the bacterium as standard samples, the other study used recombinant plasmid DNA containing *msa* gene, since recombinant plasmid containing *R. salmoninarum* gene amplifies more readily than genomic *R. salmoninarum* in a qPCR assay (Sandell and Jacobson, 2011). However, prevalence of the bacterial genome was found to be markedly higher in this study than that reported by Suzuki and Sakai (2007). In particular, samples from chum salmon derived from Sites A, F and G, and samples from masu salmon from Sites H and J showed 60%–98% of the prevalence determined in 2016.

In other recent research, we detected the *msa* gene DNA and *msa* mRNA of *R. salmoninarum* in the kidneys and surface mucus of asymptomatic chum salmon fry and juveniles in a hatchery in Hokkaido (Suzuki *et al.*, 2017). It is well established that *R. salmoninarum* cells can be transmitted horizontally as well as vertically, from adult females via gametes to offspring (Elliott *et al.*, 1989; Murray *et al.*, 1992; Okuda *et al.*, 2006). Increase

of the infection levels in the ovarian fluid can lead to rise of transmission risks and rates of the bacterium. Therefore, continuous monitoring of *R. salmoninarum* infection levels in the returning chum and masu salmon adults is quite important to take efficacious preventive measures to a decrease in transmission risks of the bacterium from the adults to their offspring.

Acknowledgements

The authors are grateful to the staff at the Hokkaido Salmon Resources Association, Japan, for their cooperation and assistance during implementation of the study.

References

- Elliott, D. G., R. J. Pascho and G. L. Bullock (1989): Developments in the control of bacterial kidney disease of salmonid fishes. *Dis. Aquat. Org.*, **6**, 201–215.
- Evenden, A. J., T. H. Grayson, M. L. Gilpin and C. B. Munn (1993): *Renibacterium salmoninarum* and bacterial kidney disease—the unfinished jigsaw. *Annu. Rev. Fish Dis.*, **3**, 87–104.
- Faisal, M. and A. E. Eissa (2009): Diagnostic testing patterns of *Renibacterium salmoninarum* in spawning salmonid stocks in Michigan. *J. Wildlife Dis.*, **45**, 447–456.
- Kasai, H., T. Nomura and M. Yoshimizu (2003): Surveillance and control of salmonid viruses of wild salmonid fish returning to the northern part of Japan, from 1976 to 2002. In [Proceedings of the 3rd FiSCUP Japan-Korea Joint Seminar on Fisheries Sciences], pp. 142–147, December 15–16, 2003, Jinju-Tongyeong, Korea.
- Maule, A. G., D. W. Rondorf, J. Beeman and P. Haner (1996): Incidence of *Renibacterium salmoninarum* infections in juvenile hatchery spring chinook salmon in the Columbia and Snake rivers. *J. Aquat. Anim. Health*, **8**, 37–46.
- Murray, C. B., T. P. T. Evelyn, T. D. Beacham, L. W. Barner, J. E. Ketcheson and L. Prosperi-Porta (1992): Experimental induction of bacterial kidney disease in chinook salmon by immersion and cohabitation challenges. *Dis. Aquat. Org.*, **12**, 91–96.
- Nomura, T. and T. Kimura (1981): Incidence of *Aeromonas salmonicida* among anadromous salmonids in Hokkaido, 1979. *Fish Pathol.*, **16**, 69–74.
- Nomura, T., M. Yoshimizu and T. Kimura (1991): Prevalence of *Aeromonas salmonicida* in chum salmon (*Oncorhynchus keta*), pink salmon (*O. gorbuscha*), and masu salmon (*O. masou*) returning to rivers in Hokkaido. *Fish Pathol.*, **26**, 139–147.
- Nomura, T., M. Yoshimizu and T. Kimura (1992): Detection of *Aeromonas salmonicida* in the coelomic fluid and kidney of mature chum salmon (*Oncorhynchus keta*), pink salmon (*O. gorbuscha*) and masu salmon (*O. masou*). *Fish Pathol.*, **27**, 69–72.
- Okuda, R., T. Nishizawa and M. Yoshimizu (2006): Infection-cycles of *Renibacterium salmoninarum* in hatcheries of masu salmon *Oncorhynchus masou* by monitoring specific antibodies. *Fish Pathol.*, **41**, 175–178.
- Pascho, R. J., D. G. Elliott and J. M. Streufert (1991): Brood stock segregation of spring chinook salmon *Oncorhynchus tshawytscha* by use of the enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody technique (FAT) affects the prevalence and levels of *Renibacterium salmoninarum* infection in progeny. *Dis.*

- Aquat. Org.*, **12**, 25–40.
- Pascho, R. J., D. Chase and C. L. McKibben (1998): Comparison of the membrane-filtration fluorescent antibody test, the enzyme-linked immunosorbent assay, and the polymerase chain reaction to detect *Renibacterium salmoninarum* in salmonid ovarian fluid. *J. Vet. Diagn. Invest.*, **10**, 60–66.
- Sandell, T. A. and K. C. Jacobson (2011): Comparison and evaluation of *Renibacterium salmoninarum* quantitative PCR diagnostic assays using field samples of Chinook and coho salmon. *Dis. Aquat. Org.*, **93**, 129–139.
- Suzuki, K. and D. K. Sakai (2007): Real-time PCR for quantification of viable *Renibacterium salmoninarum* in chum salmon *Oncorhynchus keta*. *Dis. Aquat. Org.*, **74**, 209–223.
- Suzuki, K., N. Misaka, S. Mizuno and Y. Sasaki (2017): Sub-clinical infection of *Renibacterium salmoninarum* in fry and juveniles chum salmon *Oncorhynchus keta* in Hokkaido, Japan. *Fish Pathol.*, **52**, 89–95.
- Yoshimizu, M., T. Kimura and J. R. Winton (1985): An improved technique for collecting reproductive fluid samples from salmonid fishes. *Prog. Fish-Cult.*, **47**, 199–200.
- Yoshimizu, M., T. Nomura, T. Awakura and T. Kimura (1988): Incidence of fish pathogenic viruses among anadromous salmonid in northern part of Japan (1976–1986). *Sci. Rep. Hokkaido Salmon Hatchery*, **42**, 1–20.