Seasonal Change in Serum Agglutination Titer against *Renibacterium salmoninarum* in Farmed Masu Salmon *Oncorhynchus masou*

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Abstract　Chronic bacterial kidney disease (BKD) in masu salmon, *Oncorhynchus masou* was investigated at a fish farm. In previous cases at the farm, BKD broke out concentrating in the spring-to-early-summer season. To clarify the factors of the seasonal limitation of an outbreak, the BKD infection rate of the fish was determined by isolating *Renibacterium salmoninarum* at certain intervals. In addition, the agglutination titer against *R. salmoninarum* and total immunoglobulin M (IgM) levels of the fish serum were researched as indications of host reactions against the bacteria. A seasonal change in the agglutination titer against *R. salmoninarum* was found. The characteristics of the seasonal change in the titer were a moderate increase from summer at age 0^+ until winter at 1^+, an acute decrease in spring at 1^+, a moderate recovery from summer at 1^+ until winter at 2^+, an acute decrease in spring at 2^+ and a moderate decrease until spawning. A seasonal change in the agglutination titer was also found in the fish group that was negative on the bacterial isolation. The seasonal change in the agglutination titer did not completely accord with the seasonal changes in total IgM levels or water temperature. From the characteristics of the seasonal change in the agglutination titer and the past timing of outbreaks on the farm, the occurrence of BKD was considered to be closely correlated with a decrease in the agglutination titer against *R. salmoninarum*.

Key words: masu salmon, BKD, agglutination titer

Introduction

Masu salmon, *Oncorhynchus masou*, is anadromous salmonid distributed in the Far East and an important species for fisheries in Japan (Machidori and Kato, 1984). In Hokkaido, artificial stock enhancement of this species has been performed using fish produced by pond culture as a counter plan against the trend of decreasing stock since the 1970s (Nagata, 2002).

A problem in fish culture is bacterial kidney disease (BKD) caused by the pathogen, *Renibacterium salmoninarum*. The infection has often been observed since 1973, which saw the first outbreak in Hokkaido (Awakura, 1978). Most serious characteristic of this disease is intra-ovum infection reported by Evelyn *et al*. (1984). This vertical infection (infection from parents to offspring) of *R. salmoninarum* means that the disinfection of the egg surface using povidone-iodine is not always effective. Kimura (1978) researched the features of natural outbreaks of BKD and stated that the infection tended to be chronic on farms, and sometimes caused high mortality if certain triggers were present.

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In this study, we researched the farm that had often suffered damage by BKD in order to obtain knowledge about what triggers an outbreak. In past cases at the farm, BKD mostly broke out from spring to early summer. To clarify the factors of the seasonal limitation of an outbreak, the BKD infection rate of the fish was determined by isolatimg *R. salmoninarum* at certain intervals. In addition, the agglutination titer against *R. salmoninarum* and total immunoglobulin M (IgM) levels of the fish serum were researched as indications of host reactions against the bacteria.

Materials and Methods

Fish

Masu salmon, *Oncorhynchus masou*, cultured at a farm located southwest of Hokkaido Prefecture of Japan was researched. Past outbreaks of BKD on the farm were recorded by the Hokkaido Fish Hatchery as the diagnosis document (Table 1). The fish researched in this study were hatched in 1998 (Group 1), in 1999 (Group 2) and in 2000 (Group 3). Sampling data including date, number of fish, fish age, and average body weight of fish are shown in Table 2. The fish collected at the last samplings in Group 1 (3 Oct. 2001) and Group 2 (24 Sep. 2002) were female after spawning. The water for the fish culture was drawn from a nearby river, and sometimes mixed with well water. The water temperature of the pond was measured automatically using a temperature sensor.

Table 1 Past outbreaks of bacterial kidney disease on the farm researched in this study [*1]

Date of diagnosis	Age of infected-fish	Water temperature (℃) [*2]
5 July 1988	2^+	13
29 May 1992	2^+	9
5 July 1994	2^+	15
24 July 1997	2^+	18
4 June 1999	2^+	14
2 May 2000	1^+, 2^+	8

[*1] Outbreaks of BKD were recorded in the diagnosis document of Hokkaido Fish Hatchery.
[*2] Approximate temperature of the rearing water during the outbreak.

Table 2 Sampling data

Group	Date	Fish age	Number of fish	Average weight of fish (g)
1	7 May 2001	2^+	30	407.3
1	10 Jul. 2001	2^+	30	609.8
1	3 Oct. 2001	2^+	41	703.5
2	7 May 2001	1^+	30	36.1
2	10 Jul. 2001	1^+	30	81.0
2	26 Sep. 2001	1^+	30	209.4
2	5 Feb. 2002	2^+	30	292.7
2	17 May 2002	2^+	30	508.1
2	24 Sep. 2002	2^+	40	741.4
3	10 Jul. 2001	0^+	30	1.5
3	26 Sep. 2001	0^+	30	10.1
3	5 Feb. 2002	1^+	30	25.2
3	16 May 2002	1^+	30	64.4
3	26 Aug. 2002	1^+	30	180.1
3	18 Dec. 2002	2^+	30	368.0
3	28 Mar. 2003	2^+	30	429.5

Detection of the bacteria

R. salmoninarum was detected from the fish kidney by cultivation using a agar medium based on KDM-2 (Evelyn, 1977). The medium consisted of proteose peptone No. 3 (DIFCO LABORATORIES Inc., Detroit, Michigan, U. S.) at 10 g per l, yeast extract (DIFCO LABORATORIES) at 0.5 g per l, cystein hydrochloride at 1 g per l, and agar (DIFCO LABORATORIES) at 15 g per l. This medium was supplemented with 1% of the broth medium (same component as described medium except the agar) spent for the culture of *R. salmoninarum*. The test agar plates were incubated at 15 ℃ for 4 weeks. The bacterial colony cultured on the test plate was identified by the agglutination test using an antibody against *R. salmoninarum* (Kirkegaard & Perry Laboratories Inc. Geitherburg, Maryland, U.S.).

Serum agglutination titer against *R. salmoninarum*

R. salmoninarum, strain 20-1, isolated from masu salmon reared at the farm in 1998 was cultured in order

to determine the agglutination titer of the fish serum. A liquid culture of the strain was inactivated by heating at 100 ℃ for 30 min. The cells were harvested by centrifugation at 12,000 X *g* for 10 min, and suspended with phosphate-buffered saline (PBS) at optical density (600 nm) 0.5. The serum obtained from the fish blood was serially diluted with PBS. Fifty microliters of diluted serum was mixed with the same volume of the bacterial suspension in the 96-well microplate with a rounded bottom (IWAKI, Co. Ltd., Tokyo, Japan). The agglutination titer was red after incubation at 20 ℃ for 18 h.

IgM concentration of the serum

The IgM concentration of the fish serum was measured by ELISA. A solution of rabbit IgG against masu salmon IgM (anti-masu salmon IgM) was kindly supplied from Prof. A. Hara, Graduate School of Fisheries, Hokkaido University, Japan. The protocols for the preparation of rabbit IgG against masu salmon IgM were described by Fuda *et al*. (1991).

Fifty microliters of fish serum diluted 160-fold with PBS was dropped on the wells of the ELISA plate (IWAKI) and incubated at 4℃ for 18 h. The wells of the plate were washed three times with PBS-T, PBS containing 0.05 % of Tween 20 (Bio-Rad, Inc., Hercules, California, U.S.). For blocking, 100 μl of PBS-T containing skim milk at 2 % was dispensed to each well and incubated at 4 ℃ for 6 h. The wells were washed three times with PBS-T. Fifty microliters of anti-masu salmon IgM diluted 1000-fold with PBS-T was dispensed to the wells and incubated at 20 ℃ for 18 h. The wells were washed three times with PBS-T. A HRP-labeled IgG against rabbit IgG (from goat, Bio-Rad) was diluted 2000-fold with PBS-T. Fifty microliters of the HRP-labeled IgG solution was dispensed to each well and incubated at 37 ℃ for 2 h. The well was washed five times with PBS-T. Fifty microliters of the solution for color development (0.1 M Na_2HPO_4, 0.05 M citrate, o-phenyrendiamine tetra chloride at 100 mg per l and 0.03 % of H_2O_2) was dispensed to the wells and incubated at room temperature for 15 min in the dark. Finally, 50 μl of 0.1 M H_2SO_4 was added to the wells. The optical density (absorbance) at 405 nm was measured on a microplate reader, MPR 4Ai (TOSOH Co. Ltd., Tokyo, Japan). The standard curve was conducted by the densities of the serial dilutions of a pre-measured serum supplied from the Graduate School of Fisheries, Hokkaido University, Japan.

Results

Temperature of rearing water

The water temperature of each pond was continuously recorded during the research period. The monthly averages of the temperatures at 10 a. m. are shown in Fig. 1. The water temperature changed seasonally. The maximum averages in the years (15.5℃ to 17.5℃) were recorded in August. The minimum averages in the years (2.8℃ to 3.7℃) were recorded in December or January.

Infection of bacterial kidney disease

The rates of the infected fish in each group are shown in Fig. 2. Infections were detected in Group 1 and Group 2. In Group 1, infections were detected at every three samplings. The rates of the infected fish in Group 1 were 26.7 % on 7 May 2001, 10.0 % on 10 July 2001 and 25.0 % on 3 October 2001. In Group 2, infection was detected only once (3.3 % on 5 February 2002) in six samplings. Infection was not detected in Group 3 during the research period.

Changes in serum agglutination titer against *R. salmoninarum*

The serum agglutination titer against *R. salmoninarum* was classified to < 1: 40, 1:40, 1:80, 1:160 and ≧ 1:360. The results of the serum agglutination titer are shown in Fig. 3 as the columns consisted of the percentages of the titer classes at each sampling. The titer of Group 1 decreased from May at age 2^+ until the maturation period in October. The titer of Group 2 increased from May at age 1^+ until February at age 2^+. From February at age 2^+, the titer of Group 2 decreased until maturation period of October. The titer of Group 3 tended to increase from July at age 0^+ until February at age 1^+. The titer of Group 3 decreased once in May at age 1^+, and increased again until December at age 2^+.

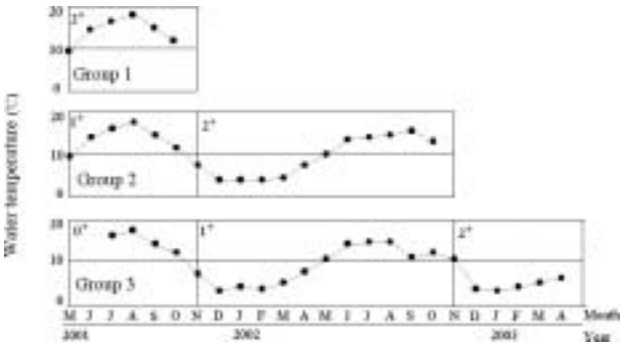

Fig. 1 The temperature of rearing water for each group. Each circle indicates the monthly average of the temperature at 10 a. m. The graphs for the groups are arrayed corresponding to the fish age.

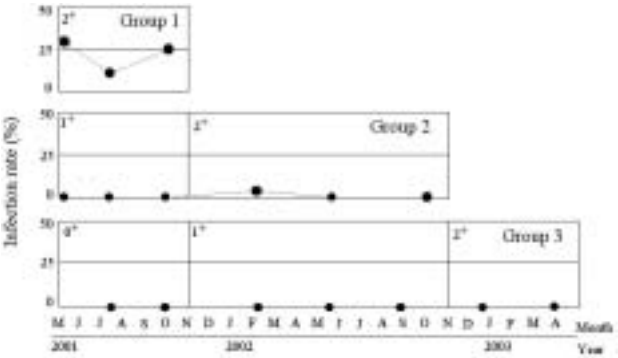

Fig. 2 Infection rate in each group. Each circle indicates the percentage of infected fish. The graphs for the groups are arrayed corresponding to the fish age (0^+, 1^+ and 2^+) and sampling date.

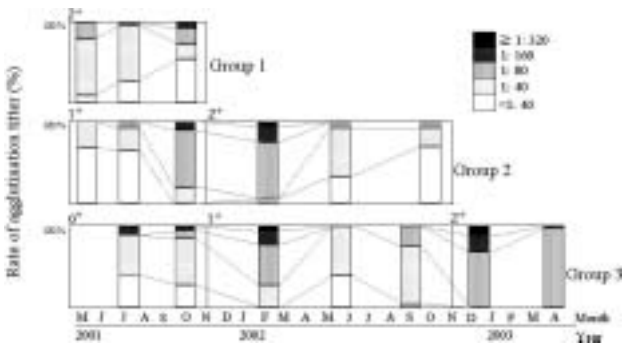

Fig. 3 Serum agglutination titer against *Renibacterium salmoninarum* in each group. The rates of titer classes are indicated as percentage columns and arrayed corresponding to the fish age (0^+, 1^+ and 2^+) and sampling date.

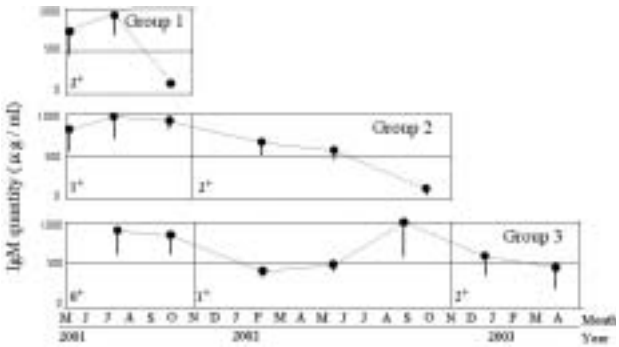

Fig. 4 IgM level in each group. Each circle indicates the average of IgM (μg per ml). Bar indicates standard deviation. The graphs for the groups are arrayed corresponding to the fish age (0^+, 1^+ and 2^+) and sampling date.

Changes in IgM concentration

The serum IgM levels of each group are shown in Fig. 4. The average serum IgM levels were 390 to 967 μg per ml except for the sampling of the post-spawned fish. The average IgM levels of the post-spawned fish (final sample in Group 1 and Group 2) were in the range of 100 to 200 μg per ml. The IgM levels of Group 1 (age 2^+) increased from May until July, and decreased until the final sampling in October. The levels of Group 2 increased from May until July at age 1^+. Then, the levels started to decrease until September at age 2^+. The IgM levels of Group 3 tended to decrease from July at age 0^+ until February at age 1^+. The levels of Group 3 increased once until August at age 1+, and decreased again until March at age 2^+.

Correlation between infection and the parameters

In Group 1, BKD infections were detected at every three samplings (Fig. 2). The agglutination titer and IgM levels of Group 1 were analyzed comparing infected fish with non-infected fish. From the compositions of the titer class percentages (Fig. 5), the titers of the infected fish were clearly higher than the titers of the non-infected fish at every sampling. The IgM data are shown in Fig. 6. The means of the IgM levels of the infected fish showed no significant difference (*t-test*) with the means of the levels of the non-infected fish at every samplings.

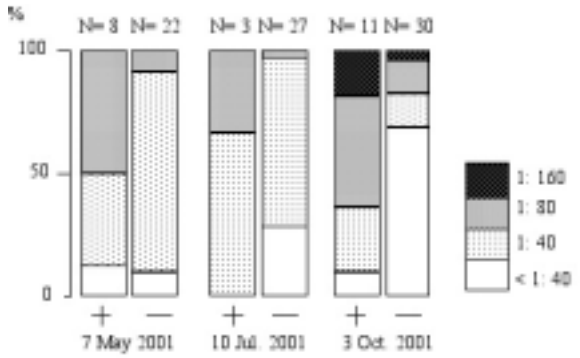

Fig. 5 Correlation between the infection and the agglutination titer. The agglutination titer on Group 1 was analyzed comparing infected fish with non-infected fish. The rates of classes of agglutination titer against *Renibacterium salmoninarum* are indicated as percentage columns. Sampling date is attached with each column. Plus and minus indicate infected fish and non-infected fish, respectively.

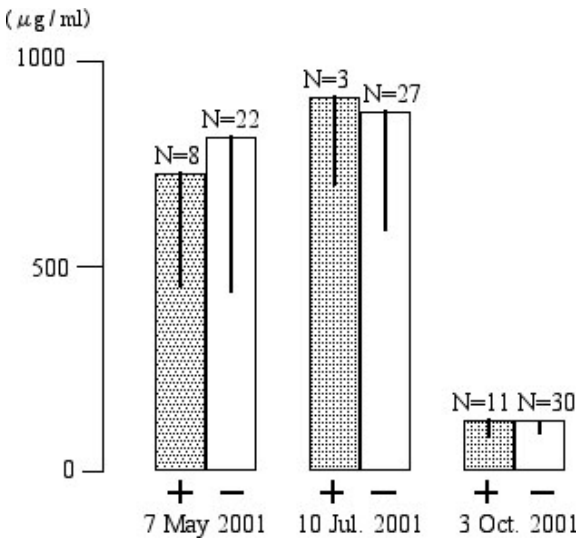

Fig. 6 Correlation between the infection and the IgM level. The IgM level on Group 1 was analyzed comparing infected fish with non-infected fish. Averages of IgM level are indicated as columns with standard deviation (bar). Sampling date is attached with each column. Plus and minus indicate infected fish and non-infected fish, respectively.

Discussion

Past outbreaks of BKD were concentrated from spring to early summer at the farm researched (Table 1). The water temperature of the term was 8 to 18℃ (Table 1). The temperature during this critical period was in the range suitable for *R. salmoninarum* growth. However, the concentration of the outbreaks cannot be explained completely by the seasonal change in temperature, because the temperature suited for bacterial growth was also recorded in the autumn.

In this study, the agglutinating reaction against *R. salmoninarum* changed seasonally (Fig. 3). This seasonal change in the agglutination titer was also found in the fish group that was negative on the bacterial isolation (Fig. 2). The characteristics of the seasonal change of the life cycle were a moderate increase from summer at age 0^+ until winter at 1^+, an acute decrease in spring at 1^+, a moderate recovery from summer at 1^+ until winter at 2^+, an acute decrease in spring at 2^+ and a moderate decrease until the spawning season. The significant point of the characteristics was assumed to be a decrease in spring.

The agglutinating reaction of fish serum against bacteria is an important defense of hosts for the exclusion of pathogens, and this is defined by the role of immunoglobulin (Kaattari and Piganelli, 1996). The elevation of agglutination titer is usually induced by contact with the bacteria. There are studies that attempted to find infected fish by ELISA detecting immunoglobulin against bacterial pathogens. Yoshimizu *et al*. (1992) suggested the application of ELISA for the prevention of flunclosis on a farm. Jansson and Lijungberg (1998) have reported the utility of ELISA by experimental infection with BKD. The humoral response of fish due to infection was clearly detected in their study. Elevation of the agglutination titer due to the infection was also found in our research (Fig. 5). However, the seasonal change in the agglutination titer found in this study indicates that the titer can change due to certain factors not correlated with the infection.

Serum IgM levels also changed seasonally (Fig. 4). The seasonal change in total IgM levels was found to have a similar trend to the change in water temperature

(Fig. 1). In the fish after spawning, IgM levels were remarkably low level (Fig. 4). IgM levels may not indicate the presence of BKD infection or activity of host defense against the bacteria, because an elevation of IgM was not observed in infected fish (Fig. 6). The seasonal change in total IgM did not completely accord with the change in the agglutination titer. A difference in the seasonal changes was observed in the winter. The agglutination maintained relatively high titers, contrary to the low IgM levels in the winter. This fact may indicate that the agglutination titer (the levels of IgM for the bacterial agglutination) changes seasonally due to certain factors that are different from the total IgM levels.

Seasonal changes in immune response were reported in some fish species (Burreson and Frizzel, 1986; Nakanishi, 1986). For salmonid fish, Lopez- Fierro *et al*. (1994) reported that total IgM and the agglutination titer against *Yersinia rucheri* had low values in the winter in farmed rainbow trout. Tatner (1996) stated that the natural change in the immunological parameters of the fish cannot be concluded simply due to the change in water temperature, because a seasonal change in immunological reaction was also observed in the fish reared at a constant temperature. Fish immunity may be influenced by hormone levels that change through lifecycle events. In coho salmon, serum cortisol levels greatly elevated due to acute stress during parr-smolt transformation (Barton *et al*., 1985). Masu salmon is also a salmonid that undergoes parr-smolt transformation in spring at age 1^{+} (Kubo, 1980). Nagae *et al*. (1994) investigated the immuno-modulation by cortisol, and found that the oral administration of cortisol resulted in a decrease of IgM levels in masu salmon.

In this study, we found seasonal changes in the agglutination titer against *R. salmoninarum*. We consider that chronic BKD may cause high mortality when the host defenses including the agglutination titer decrease. On the farm researched, BKD may break out in spring or early summer because of low levels of host defense against bacteria and a suitable temperature for bacterial growth. In addition, the disease may not cause high mortality in autumn because the levels of defense are high.

Acknowledgements

The authors would like to express deep gratitude to Prof. Akihiko Hara at the Graduate School of Fisheries, Hokkaido University who advised us regarding the ELISA techniques and provided us with the antibody.

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和文摘要

池中飼育サクラマスの細菌性腎臓病病原菌（*Renibacterium salmoninarum*）に対する血中凝集抗体価の季節的変動

畑山　　誠・三浦　宏紀

ある養魚施設のサクラマスに発生する細菌性腎臓病（BKD）について調査した．調査対象とした養魚施設は1980年代後半からたびたびBKDの発生がみられ，その病歴記録により，比較的高率な死亡を伴うBKDの発生は春から初夏に限定されることがわかった．発生の季節が限定されることについて，その原因を明らかにすることは慢性的BKDの病態を知る上で重要と考えられる．病勢の季節的変動の指標として，経時的にサクラマス腎臓組織からの原因菌（*R. salmoninarum*）の検出を行い，感染率の推移を調査した．同時にBKDに対する抗病性の指標としてサクラマス血液の病原菌に対する凝集抗体価の測定とトータルなimmunoglobulin M (IgM)の定量を行った．調査によりサクラマス血液の原因菌に対する凝集抗体価は年齢1^+, 2^+ともに春季に大きな落ち込みがあることが推察された．この凝集抗体価の季節的変動は原因菌が検出されなかった群においても確認された．これらの結果より慢性的BKDが春から初夏に大量死をもたらすことについて，サクラマスの凝集抗体価等の抗病性の低下が引き金になっていることが考えられた．凝集抗体価の変動要因は不明であるが少なくともIgM量の季節変動や水温変化と連動するものではないことが明らかとなった．