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Detection of antibody against *Aeromonas salmonicida* in the serum of salmonid fish by the enzyme linked immunosorbent assay

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Enzyme linked immunosorbent assay (ELISA) using the anti-masu Ig rabbit serum was applied to detect the antibody against *Aeromonas salmonicida* in the sera of salmonid fish. This ELISA method could detect the antibody in sera of masu (*Oncorhynchus masou*), chum (*O. keta*) and coho salmon (*O. kisutch*) immunized by *A. salmonicida* and could be applied to detect the antibody against *A. salmonicida* in sera of matured masu, pink (*O. gorbuscha*), chum, and kokanee salmon (*O. nerka*). In 1989, 89 masu and 250 chum salmon, and 59 kokanee were examined to detect antibody against *A. salmonicida* by ELISA and agglutination test. The number of the fish showing the positive reaction by ELISA, agglutination test and isolation of *A. salmonicida* were 262, 48, and 33, respectively. In 1990, 200 masu and kokanee salmon fry released from hatchery to river were tested and antibody against *A. salmonicida* was detected from 111 fish by ELISA, and the incidence of the fish showing the positive reaction was ranged from 4 to 90%.

During the past decade, a systematic etiological study was performed to establish control measures for furunculosis in Hokkaido (Nomura and Kimura, 1981; Nomura *et al.*, 1983, 1991a, b). In Hokkaido, outbreaks of furunculosis had been reported in chum salmon (*Oncorhynchus keta*) by Nishino (1967), in masu salmon (*O. masou*) and pink salmon (*O. gorbuscha*) by Kimura (1969a, b, 1970) during its maturation in holding ponds. Nomura *et al.* (1991a, b) isolated *Aeromonas salmonicida* in high incidence from the kidney of matured anadromous chum, pink and masu salmon which showed no apparent clinical signs of furunculosis.

Serodiagnostic techniques are particularly applied to the diagnosis and surveillance of *A. salmonicida* because of the fact that the organism is not readily isolated from the fish in the asymptomatic carrier phase of infection (Weber and Zwicker, 1979; Krantz and Heist, 1970). It has become increasingly important to measure or monitor the antibody responses of fish. A variety of serological methods are available for this purpose, including the often used agglutinin titration with whole bacterial cells as antigens, passive hemagglutination using red cell sensitized with bacterial fragment or product, and the more recently developed radioimmunoassays and enzyme-liked reaction (Engvall and Perlmann, 1972).

Weber and Zwicker (1979) studied the antibody titer against *A. salmonicida* in the serum of spawned Atlantic salmon broodstock from the Restigouche River, Miramichi R. and Margaree R. by agglutination method. However, the agglutinins were not detected in fingerling trout less than a year old. Nomura *et al.* (1991c) reported that the agglutinin titer against *A. salmonicida* in sera of mature chum, pink, and masu salmon showed variability within the species.

An enzyme-linked immunosorbent assay (ELISA) was developed to detect antibody against *A. salmonicida* in rainbow trout immunized with the formalin-killed bacteria (Kodama *et al.*, 1985). Watanabe and Suzuki (1986) prepared a rabbit antiserum against rainbow trout immunoglobulins (Ig) and this antiserum reacted

with the Ig of some other species of genus *Oncorhynchus*. In the present report, we prepared an antiserum against masu salmon Ig and developed an ELISA by using the antiserum for detection of antibody against *A. salmonicida* in sera of wild salmonid fish.

Materials and Methods

Antigen Preparation

Aeromonas salmonicida subsp. *salmonicida* (ATCC 14174) was cultured in trypticase soy broth (TSB; BBL) at 25°C for 48 h. The bacterial culture was centrifuged at 1,800×*g* for 20 min and washed with PBS 3 times. After washing, the bacterial suspension was heated at 100°C for 30 min and again centrifuged at 1,800×*g* for 20 min. The cells were resuspended in PBS (with 0.1% NaN_3) and used for heat-killed cell antigen. The supernatant was used for the heat extract antigen.

Preparation of Rabbit Antiserum Against A. salmonicida

A New Zealand white rabbit was intrapenously injected with the heat killed cell antigen and then boosted by intramuscularly or hypodermic injection with an emulsion of heat killed cell suspension and Freund's complete adjuvant (1:1). The rabbit was bled 7 day after the last injection. Serum was collected, sterilized by membrane filtration, inactivated at 56°C for 30 min and stored at −80°C until used.

Preparation of Rabbit Antiserum against Masu Salmon Immunoglobulin

A rabbit antiserum against masu salmon Ig was prepared and purified according to the method of Watanabe and Suzuki (1986). Briefly, masu salmon (*O. masou*) was immunized with 10^{10} cells of rabbit red blood cells (RaRBC) by four intraperitoneal injections at an interval of 10 days and the serum was collected 7 days after the last injection. Then the serum was incubated with 10^9 cells of RaRBC suspended in veronal buffer supplemented with 0.02 M EDTA pH 7.5 to prevent complement conjugation. After 24 h reaction at 4°C, the agglutinated RaRBC was washed 3 times with the veronal buffer and intravenously injected 4 times to rabbit at an interval of 10 days. The blood was collected 7 days after the last injection, and the serum was inactivated and stored as described above.

Measurement of Agglutination Titer

A half ml of serial 2 fold dilutions of rabbit or fish antiserum with PBS was mixed with same volume of the heat killed cell antigen, the concentration of which had been adjusted to McFarland No. 3, and incubated at 37°C for 2 h and 4°C overnight. Agglutination titer was read by naked eye.

Effect of Antigens on ELISA

Fifty and 100 μl of the heat extract antigen, and 50 μl of heat killed cell antigens (10^8 cell/ml) were placed in each well of 96 well plate for ELISA (Corning) and incubated at 37°C overnight. Each well was washed 3 times with PBS-Tween, and 50 μl of 2% skim milk was added in each well, incubated at 37°C for 1 h, and then 50 μl of anti-*A. salmonicida* rabbit serum was added in each well. After incubation at 37°C for 30 min, the plate was again washed 3 times. Fifty μl of a peroxidase conjugated swine antiserum against rabbit immunoglobulin (Dakkopatts; diluted 1:100 in PBS-Tween) was added in each well. The suspension was incubated at 37°C for 30 min and plates were washed 3 times again. Fifty μl of 3, 3-diaminobenzidine-tetrahydrochloride in 0.05 M Tris-buffer pH 8.2 (5 μg/10 ml) was added in each well. After 10 min, the reaction was stopped with 4 N HCl. And the absorbance was measured by spectophotometer (MTP-22; Corona) at a wavelength of 492 nm.

ELISA to Detect Antibody against A. salmonicida *in Fish Serum*

Fifty μl of the heat killed cell antigen was placed in each well of 96 well plate and incubated at 37°C overnight, and then washed 3 times with PBS-Tween. In each well, 50 μl of 2% skim milk was added and incubated at 37°C for 1 h. Fifty μl of fish serum was added, and incubated at 37°C for 30 min. The plate was washed 3 times and 50 μl of the rabbit anti-masu salmon Ig (diluted 1:200 in PBS-Tween) was added in each well and then absorbance was determined by the ELISA method described above.

Base Line Determination for ELISA

Blood of 30 masu salmon returning to the Shiribetsu River (the average body weight was 2,545 g) were collected from the caudal vein, and centrifuged at 1,800×*g* for 20 min. Sera were employed to determine the agglutination titer against *A. salmonicida* and ELISA absorbance was determined by the ELISA method described above. To determine the base line of the ELISA, sera from 10 fish were mixed with the same volume of pelleted heat-killed *A. salmonicida* antigens, incubated at 4°C overnight to remove the specific antibody in the sera and centrifuged at 1,800×*g* for 5 min, and then ELISA absorbance was determined.

Immunization of Fish

Thirty of masu salmon cultured in Chitose Hatchery of Hokkaido Salmon Hatchery (the average body weight was 46.6 g) and specific pathogen free (SPF) chum and coho salmon (*O. kisutch*) fry cultured in our laboratory (the average body weight was 9.2 g and 25.1 g, respetively) were injected with 10^8 cells of heat killed *A. salmonicida* antigen. Before immunization, 2 weeks and 4 weeks after immunization, the sera were collected and the agglutination titer and ELISA titer were determined. Serum was diluted and maximum dilution rate to show ELISA absorbance more than 0.010 at wave length of 492 nm was measured. This maximum dilution was considered as ELISA titer.

Detection of Antibody against A. salmonicida *in Sera of Matured or Fry Stage of Salmonid Fish by ELISA*

From September to November in 1989, 89 of masu, 250 of chum, and 59 of kokanee salmon (*O. nerka*) were collected from 10 catching stations of Hokkaido Salmon Hatchery, namely, Chitose R., Kenebetsu R., Shari R., Shibetsu R., Shikotsu Lake, Shokanbetsu R., Tohoro R., Tokushibetsu R., Teshio R., and Yuurappu R. In October 1990, 240 of chum, 283 of masu, and 71 of pink salmon were collected from 8 catching stations of Hokkaido Salmon Hatchery, namely, Furen R., Nishibetsu R., Shari R., Shibetsu R., Shiribetsu R., Shokotsu R., Tokachi R. and Tokushibetsu R. Blood samples were taken from the caudal vein and sera were collected as described above. The serum was diluted 1:5 in PBS-Tween and used for antibody detection against *A. sal-*

Table 1. Comparison of agglutination titers and ELISA titers of an anti-*A. salmonicida* rabbit serum, and the effect of the antigen on ELISA

Dilution	Agglutination test	ELISA absorbance (OD×1,000) Volume of antigen		
		50 μl		100 μl
		Heat killed	Heat extract	Heat extract
1: 100	++*1	55.0	68.5	77.0
1: 200	++	60.0	57.0	75.0
1: 400	++	64.5	55.0	62.0
1: 800	++	64.0	56.0	77.0
1: 1600	+*1	60.5	53.0	83.5
1: 3200	+	58.0	61.0	90.0
1: 6400	—	50.0	51.0	64.5
1: 12800	—	24.5	18.0	25.5
1: 25600	—	0.0	1.0	6.5
1: 51200	—	0.0	2.0	5.0
1: 10240	—	0.0	0.0	1.5
Control	—	0.0	0.0	0.0
Antibody titer	1: 3200	1: 12800*2	1: 12800*2	1: 12800*2

*1 ++ An agglutinated pellet could be observed.
+ Slight agglutination could be observed.

*2 Dilutions at which the ELISA absorbance was more than 0.010.

monicida by ELISA using the anti-masu salmon Ig rabbit serum or agglutination test. The serum samples that showed ELISA absorbance of more than 0.010 at a wave length of 492 nm was considered as positive.

In October 1990, 170 of masu and 30 of kokanee salmon fry were collected from 4 hatcheries of Hokkaido Salmon Hatchery, namely, Chitose H., Shari H, Ichani H. and Shibetsu H. Serum preparation and detection of antibody by ELISA were done as described as above.

Isolation of A. salmonicida

Nutrient agar (NA; Eiken) was used for isolation of *A. salmonicida* from the kidney of fish. Isolation was carried out according to the method described by Nomura *et al.* (1991a).

Results

Comparison of Agglutination Titer and ELISA Titer, and Effect of the Antigen on ELISA

Agglutination titer and ELISA titer of rabbit anti-*A.salmonicida* serum were determined. As shown in Table 1, the agglutination titer was 1: 3200 and the ELISA titer was 1: 12800. For the ELISA, two kinds of antigens and two volumes of antigens were tested. Endpoint of the reaction was clear when heat-killed cell antigen was used. It was suggested that the absorbance above 0.010 might show the positive reaction. Volume of the antigen did not have much effect on the results of ELISA.

Table 2. Agglutination titer and ELISA absorbance of non-treated and absorbed sera of masu salmon by heat-killed *A. salmonicida* cell antigen

Fish No.	ELISA absorbance*1 of sera		Agglutination titer
	Non-treated	Absorbed	
1	6	6	<1: 4
2	3	5	<1: 4
3	12	4	1: 4
4	0	0	<1: 4
5	0	3	<1: 4
6	11	5	1: 4
7	3	0	<1: 4
8	0	3	<1: 4
9	0	3	<1: 4
10	2	1	<1: 4

*1 OD (×1,000).

Base Line Determination for ELISA

Results of the measurement of agglutination titer against *A. salmonicida* in sera of masu salmon collected from Shiribetsu River and ELISA absorbance of these non-treated and absorbed sera by *A. salmonicida* antigen are listed in Table 2. Although the sera absorbed with *A. salmonicida* antigen showed the ELISA absorbance from 0.000 to 0.006 at a wave length of 492 nm, ELISA absorbance of two non-treated sera which showed the agglutination titer (1: 4) was 0.011 and 0.012. ELISA absorbance of the sera from 20 masu salmon collected in the same river are shown in Table 3. ELISA absorbance of the sera which had agglutination titer (1: 4) was more than 0.010. From these

Table 3. ELISA absorbance and agglutination titer in sera against *A. salmonicida* antigen, and results of isolation of *A. salmonicida* from masu salmon collected from Shiribetsu River

Fish No.	ELISA absorbance (OD×1,000)	Agglutination titer	Isolation of *A. salmonicida*
1	2	<1: 4	—
2	8	<1: 4	—
3	7	<1: 4	—
4	8	<1: 4	—
5	4	<1: 4	—
6	4	<1: 4	—
7	13	1: 4	—
8	3	<1: 4	—
9	3	<1: 4	—
10	6	<1: 4	—
11	3	<1: 4	—
12	3	<1: 4	—
13	ND*1	ND	—
14	1	<1: 4	—
15	8	<1: 4	—
16	5	<1: 4	—
17	5	<1: 4	—
18	2	<1: 4	—
19	4	<1: 4	—
20	1	<1: 4	—
Positive	1/19*2	1/19	0/20

*1 Not determined.

*2 ELISA absorbance more than 0.01.

Table 4. Agglutination titer and ELISA titer in sera of immunized masu salmon against *A. salmonicida* cultured in Chitose Hatchery

Fish No.	Before		2 weeks after		4 weeks after	
	Agg. T.*1	ELISA T.*1	Agg. T.	ELISA T.	Agg. T.	ELISA T.
1	<1:5	1:40	<1: 5	1: 40	<1: 5	1:160
2	<1:5	1:40	1:10	1:160	1:160	1:320
3	<1:5	1:20	<1: 5	1: 10	1: 80	1:160
4	<1:5	1:40	1:80	1:320	1: 80	1:160
5	<1:5	1:40	1:80	1:320	1: 40	1:160
6	<1:5	1:40	1:20	1: 80	1: 40	1: 80
7	<1:5	1:40	<1: 5	1: 20	1: 40	1:160
8	<1:5	1:20	<1: 5	1: 40	1:160	1:320
9	<1:5	1:40	<1: 5	1: 40	ND*2	ND
10	<1:5	1:40	1:10	1: 80	ND	ND

*1 Agglutination titer and ELISA titer.
*2 ND: Not determined.

Table 5. Agglutination titer and ELISA titer in sera of immunized SPF chum salmon against *A. salmonicida*

Fish No.	Before		2 weeks after		4 weeks after	
	Agg. T.*1	ELISA T.*1	Agg. T.	ELISA T.	Agg. T.	ELISA T.
1	<1:5	<1:5	<1: 5	1: 40	1:160	1:320
2	<1:5	<1:5	1: 5	1: 80	1: 80	1:160
3	<1:5	<1:5	<1: 5	1: 40	1: 80	1:160
4	<1:5	<1:5	1: 5	1: 80	1:160	1:320
5	<1:5	<1:5	1: 5	1:160	1:320	1:320
6	<1:5	<1:5	1: 5	1:160	1:320	1:320
7	<1:5	<1:5	<1: 5	1: 40	1:160	1:320
8	<1:5	<1:5	1: 5	1: 80	1:160	1:320
9	<1:5	<1:5	<1: 5	1:160	1:160	1:320
10	<1:5	<1:5	1:10	1:160	1:160	1:320

*1 Agglutination titer and ELISA titer.

Table 6. Agglutination titer and ELISA titer in sera of immunized SPF coho salmon against *A. salmonicida*

Fish No.	Before		2 weeks after		4 weeks after	
	Agg. T.*1	ELISA T.*1	Agg. T.	ELISA T.	Agg. T.	ELISA T.
1	<1:5	<1:5	<1: 5	1: 80	1: 40	1:160
2	<1:5	1:5	<1: 5	1: 40	1: 80	1:320
3	<1:5	1:5	<1: 5	1: 80	1: 80	1:160
4	<1:5	<1:5	<1: 5	1: 40	1:320	1:320
5	<1:5	<1:5	1: 5	1: 40	1: 80	1:320
6	<1:5	<1:5	1: 5	1:160	1: 80	1:320
7	<1:5	<1:5	1: 5	1:160	1: 80	1:320
8	<1:5	<1:5	1: 5	1: 80	1:160	1:640
9	<1:5	<1:5	<1: 5	1:160	1:320	1:320
10	<1:5	<1:5	1:10	1:320	1:320	1:640

*1 Agglutination titer and ELISA titer.

Table 7. Detection of the antibody against *A. salmonicida* in sera of matured masu, chum, and kokanee salmon collected from 12 catching stations in 1989 by ELISA, agglutination test, and results of isolation of *A. salmonicida*

Sampling		Species of fish	Number of the fish			
Place	Date		Employed	Positive reaction by		Isolation positive
				ELISA*1	Agg. T.*1	
Shari R.	Sep. 12	masu salmon	30	3	0	0
Chitose R.	Sep. 14	masu salmon	30	25	0	8
Shibetsu R.	Sep. 14	masu salmon	29	20	0	0
Chitose R.	Oct. 16	chum salmon	20	7	0	0
Teshio R.	Oct. 17	chum salmon	30	29	8	11
Tokushibetsu R.	Oct. 18	chum salmon	30	29	12	1
Shokanbetsu R.	Oct. 19	chum salmon	30	21	8	0
Shikotsu L.	Oct. 26	kokanee	30	9	ND*2	0
Kenebetsu R.	Oct. 31	kokanee	29	18	ND	0
Tohoro R.	Nov. 2	chum salmon	60	22	8	13
Yuurappu R.	Nov. 9	chum salmon	22	21	8	0
Yuurappu R.	Nov. 30	chum salmon	58	58	4	0

*1 ELISA and agglutination test, to show the OD more than 0.010.
*2 Not determined.

Table 8. Detection of the antibody against *A. salmonicida* in sera of matured masu, chum, and pink salmon collected from 11 catching stations in 1990 by ELISA and results of isolation of *A. salmonicida*

Sampling		Species of fish	Number of the fish		
Place	Date		Employed	Positive reaction by ELISA*1	Isolation positive
Shibetsu R.	Sep. 10	masu salmon	60	0	0
Shibetsu R.	Sep. 10	chum salmon	60	0	0
Nishibetsu R.	Sep. 11	masu salmon	33	8	1
Shari R.	Sep. 12	masu salmon	60	6	1
Tokushibetsu R.	Sep. 17	masu salmon	60	0	0
Tokushibetsu R.	Sep. 17	chum salmon	60	0	0
Tokushibetsu R.	Sep. 17	pink salmon	60	0	0
Shiribetsu R.	Sep. 21	masu salmon	60	3	1
Tokachi R.	Sep. 27	chum salmon	60	4	19
Shokotsu R.	Sep. 27	pink salmon	11	0	0
Shiribetsu R.	Sep. 28	chum salmon	30	0	0

*1 ELISA absorbance more than OD 0.010.

Table 9. Detection of the antibody against *A. salmonicida* in sera of masu and kokanee salmon fry cultured in 4 hatcheries collected in 1990 by ELISA

Sampling place	Species of fish	Number of the fish	
		Employed	Positive reaction by ELISA
Chitose H.	masu salmon	102	91
Shari H.	masu salmon	20	3
Ichani H.	masu salmon	26	1
Chitose H.	kokanee salmon	30	14
Shibetsu H.	masu salmon	22	2

results, ELISA absorbance indicating the antibody specific reaction was estimated to be 0.006 or more. Therefore, the specimens showing the ELISA absorbance more than 0.010 was determined to be positive.

Detection of Antibody against A. samonicida *in Sera of Immunized Fish by ELISA*

The agglutination titers and ELISA titers in the sera of masu salmon cultured on Chitose Hatchery before immunization, 2 weeks, and 4 weeks after immunization are listed in Table 4. Although, agglutination titer was not detected in sera of masu salmon before immunization (<1: 5), the ELISA titers were 1: 20 to 1: 40. Two weeks after immunization, 5 fish showed the agglutination titers ranging from 1: 10 to 1: 80, and the ELISA titers ranging from 1: 80 to 1: 320. Four weeks post immunization, the agglutination titers and the ELISA titers increased more and titer reached <1: 5 to 1: 160 and 1: 80 to 1: 320, respectively.

In the case of SPF chum and coho salmon fry reared in our laboratory immunized with *A. salmonicida*, agglutination titer and ELISA titer could not be detected, except for 2 fish of coho salmon, before immunization (Tables 5 & 6). After 2 weeks, 6 fish of chum salmon and 5 of coho salmon showed agglutination titers ranging from 1: 5 to 1: 10, and all fish showed the ELISA titetrs ranging from 1: 40 to 1: 320. After 4 weeks, all fish showed agglutination titers and ELISA titers from 1: 40 to 1: 320 and 1: 160 to 1: 640, respectively. From these results, ELISA was more sensitive than agglutination test to determine specific fish antibody.

Detection of Antibody against A. salmonicida *in Sera of Wild Matured Salmonid Fish by ELISA*

Detection of antibody against *A. salmonicida* in sera of wild mature masu, pink, chum, and coho salmon by ELISA method was carried out. Results of detection of antibody by ELISA and agglutination test are shown in Tables 7 and 8. The results of isolation of *A. salmonicida* from these fishes are also shown in the same tables.

In 1989, 89 matured masu salmon collected from 3 catching stations were tested. The number of the fish showing positive reaction by ELISA were 48 but in agglutination test all fish showed negative reaction. *A. salmonicida* was isolated from 8 fish collected from Chitose River. In each of 30 matured chum salmon collected from Teshio River and Tokushibetsu River, 29 fishes from both rivers showed positive reaction, and 8 from Teshio R. and 12 from Tokushibetsu R. showed the positive agglutination reaction. Also *A. salmonicida* was isolated from 11 fish of Teshio R. and 1 fish from Tokushibetsu River. In a total of 250 chum salmon, collected from 7 catching stations, 187 fish showed positive reaction by ELISA and 48 fish showed positive agglutination reaction. *A. salmonicida* was isolated from 11 fish from Teshio R., a fish from Tokushibetsu R., and 13 fishes from Tohoro R. In 59 Kokanee salmon from 2 catching stations, 27 fish showed the positive reaction by ELISA method. From these results, it is assumed that ELISA was more sensitive to determine the incidence of infection or affects of *A. salmonicida* than agglutination test or culture method.

In 1990, 273 masu salmon, 210 chum salmon and 71 pink salmon were collected from 5, 4 and 2 rivers, respectively. The number of the fish showing the positive reaction by ELISA and isolation of *A. salmonicida* were 17 and 3 in masu salmon, 4 and 19 in chum salmon, and 0 and 1 in pink salmon, respectively.

Detection of Antibody against A. salmonicida *in Sera of Salmonid Fry by ELISA*

Detection of antibody against *A. salmonicida* in sera of masu and kokanee salmon fry released from hatchery to river was carried out and the number of fish showing positive reaction by ELISA are listed in Table 9. Antibody against *A. salmonicida* was detected in the sera of 97 masu salmon collected from 4 hatcheries and 14 kokanee salmon from one hatchery. Detection rate ranged from 4 to 89% (Table 9).

Discussion

Conventional methods for antibody estimations in fish, such as serum agglutination and haemagglutination, are only satisfactory when the antibody levels are relatively high, for example, after i. p. injection of vaccine (Sakai and Kimura, 1985). To detect a less pronounced antibody

response or to reveal minor modulations of the immune response due to environmental disturbances, more sensitive methods would be requested (Thuvander *et al.*, 1987). The ELISA technique has previously been used in several studies of the humoral antibody response in fish as it is a sensitive and inexpensive method which permits rapid screening of large numbers of serum samples (Roberson, 1981; McArthur and Sengupta, 1982; Bortz, 1984; Chart *et al.*, 1984; Cossarini-Dunier, 1985; Hamilton *et al.*, 1986).

From the result of a preliminary study for the ELISA method which used an rabbit anti-*A. salmonicida* serum, the end point of ELISA absorbance was clear when heat-killed cell antigen was used (Table 1). The volume of the antigen, between 50 and 100 μl was not effected on ELISA (Table 1). Fixing the antigen with the glutalaldehyde (2%) effected on the specificity of the result of ELISA and temperature for fixation, 60°C and 37°C, did not effect on the result of ELISA (data not shown). Skim milk was better than FBS to block the binding site of the plate, and also concentrations of the antigen which diluted from 21 to 24 were not effected on ELISA (data not shown). In this study, we used the 50 μl of heat killed cell (10^8 cells/ml) for antigen and fixed by 37°C overnight.

To determine the base line for ELISA, we compared the ELISA absorbance of non-treated and absorbed sera by *A. salmonicida* antigen. Although the sera absorbed by *A. salmonicida* antigen showed the ELISA absorbance from 0.000 to 0.006, ELISA absorbance of two non-treated sera which showed an agglutination titer (1 : 4) was 0.011 and 0.012, respectively (Table 2). ELISA absorbance of another masu salmon sera which had an agglutination titer showed the absorbance more than 0.011 (Table 3). From these results, ELISA absorbance to show the antibody specific reaction was considered to be 0.006 or more and the specimens to show the ELISA absorbance more than 0.010 was decided to be positive.

Then, this ELISA system using the rabbit anti-masu salmon Ig serum was applied to detect the antibody in sera of immunized masu and coho salmon. ELISA method could detect antibody titer in sera of masu salmon, and the titer was increased by immunization (Table 4). This anti-masu salmon Ig rabbit serum showed the cross reaction to chum and coho salmon Ig (Tables 5 and 6) and the antibody titer in sera of chum and coho salmon could be detected and the ELISA titer increased by immunization. The sera from masu salmon before immunization showed positive reactions by ELISA; outbreaks of furunculosis occurred among the masu salmon in the farms this year and fish employed might have been infected weakly by *A. salmonicida*.

Again, we tried to collect the sera from mature masu, chum, pink, and kokanee salmon and applied ELISA method to detect the antibody against *A. salmonicida*. In 1989, 89 mature masu, 250 chum, and 59 kokanee salmon were collected from 12 catching stations in Hokkaido. This ELISA method could detect the antibody in sera of masu, chum, and kokanee salmon (Table 7). In 1990, this study was repeated again and confirmed that it could detect the antibody against *A. salmonicida* (Table 8). In the same year, 170 masu and 30 kokanee salmon fry collected from 4 hatcheries were tested. Antibody against *A. salmonicida* were detected from 97 masu and 14 kokanee salmon. In this year, in Chitose Hatchery had an outbreak of furunculosis, and the fish which had an antibody against *A. salmonicida* might survive and become a carrier of *A. salmonicida* (Table 9).

Accordingly, McCarthy (1983) postulated that if stressful conditions were absent, resident fish might become carriers without developing clinical signs of disease. Results in this study indicated that the possibility of *A. salmonicida* infection to salmonid fry during the culture in pond before it released to the ocean. The carrier fry may progress in to disease during its adaptation from river to sea or growth in sea (Ezura *et al.*, 1984).

In this study, we were able to evaluate the ELISA method to detect the antibodies against *A. salmonicida* in sera of mature salmon. We used the rabbit anti-masu salmon Ig serum which showed cross reaction with other Ig of *Oncorhynchus* species. It is concluded that ELISA system is very useful for an epizoological study of furunculosis and also has higher sensitivity compared with agglutination test.

The sensitivity of the ELISA was several times higher than that of agglutination test to determine specific antibody. Earlier experiments revealed

that fish naturally infected with *A. salmonicida* developed only very low titer of agglutinating antibody (Krantz and Heist, 1970; Weber and Zwicker, 1979). Kodama *et al.* (1985) reported that ELISA had a high sensitivity, specificity and simplicity to detect immunized rainbow trout antibody against *A. salmonicida.* From those reasons the ELISA is considered to be a practical and sensitive tool for screening large number of serum samples of wild fish with low antibody titres.

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