The Assessment of the Health Condition of Salmonids by Non-Specific Haemolytic (SH_{50}) Activity of Serum

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Normal salmonid sera were assayed for spontaneous haemolytic activity, SH_{50} activity, against washed goldfish red blood cells. When juvenile coho salmon *Oncorhynchus kisutch* and masu salmon *O. masou* were starved for 10 to 20 days and injected with *Aeromonas salmonicida* and *Vibrio anguillarum* intraperitoneally, a correlation between the decrease in the SH_{50} activity and the increase in severity of starvation and diseases was evidently represented. A similar correlation was followed by naturally diseased salmonids. The SH_{50} activity reflected the alteration in the state of health. Also, a considerable difference in the SH_{50} activity was shown by parr-smolt transformation of masu salmon. A possibility of the SH_{50} activity as a parameter of fish health assessment is discussed.

A simple reliable parameter of the state of health of natural and farmed fish is desirable but is not yet available. Blood parameters which involve whole blood analysis,¹⁾ plasma or serum analyses,²⁾ and serum enzyme activities³⁾ have been investigated. However, these techniques have not yet been adopted because of great inter- and intra-species variability. Standardization of such methods for fish have proved very difficult, particularly with samples collected in the field.

In recent years, serum and skin mucus have attracted interest as materials for the assessment of the health of fishes.⁴⁾ Analysis of mucus is still not sufficiently reliable as a parameter of health assessment despite its ease of collection.^{5, 6)}

There is a non-specific (specific antibody-independent) haemolytic activity against heterologous erythrocytes in the normal sera of a wide taxonomic range from cyclostomes to teleosts.⁷⁾ The present study was carried out in an attempt to measure the non-specific haemolytic activity in fish sera and to relate this activity to state of health of salmonids.

Materials and Methods

Fish

Juvenile (yearlings, weight range 40 to 70 g) coho salmon *Oncorhynchus kisutch* and masu salmon *O. masou*, which had been raised in the Hokkaido Fish Hatchery, were used. The fish were transferred and held for several weeks in laboratory

aquaria kept by supplying well water at 10°C. Unless otherwise indicated, they were daily given commercial dry pellet food. The fish showed no sign of disease. Naturally diseased yearlings of coho salmon and masu salmon were carried in the laboratory from fish farms and immediately used for serum collection and for the investigation of disease and causative agents.

Serum Collection

Blood was collected by caudal peduncle amputation. Blood samples were allowed to stand at room temperature and the clots removed by centrifugation at 1,000×g for 5 min at 4°C. Sera were stored either temporarily at 5°C or -80°C until use.

Haemolytic Activity of Serum

Spontaneous (antibody-independent) haemolytic activity, " SH_{50} " of fish serum was titrated at 30°C for 60 min with goldfish red blood cells (GFRBC) washed with physiological saline (0.85% NaCl). Details of the method and unit used have been reported by SAKAI.⁸⁾ GVB⁺⁺ (isotonic veronal-buffered saline, pH 7.5, containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$) was used as a diluent for the haemolytic assay through the experiments.

Culture of Bacteria

Aeromonas salmonicida (subsp. *salmonicida*) strain A-7301 and *Vibrio anguillarum* strain Vco-1

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were selected. The two strains had been isolated from diseased salmonids and were passed using masu salmon and coho salmon before use. The strains were cultured on nutrient agar slopes at 20°C for 3 days and then transferred to nutrient broth (pH 7.4). After 45 h at 20°C on an orbital shaker, the cells were harvested and washed twice with sterile physiological saline and recovered by centrifugation at $6,000 \times g$ for 15 min at 4°C. The cell density was adjusted to 10^7 viable cells per ml with saline. The moiety of the cells was treated with 0.5% formaldehyde in physiological saline and resuspended at a cell density of 1 mg wet cells per ml with the saline (without formaldehyde). Coho salmon and masu salmon each received 0.1 ml viable cell or formalin-killed cell suspension intraperitoneally (*i.p.*). The isolation of injected bacteria was attempted using the kidneys of individuals sacrificed for serum collection. Nutrient agar plates were used for the isolation and viable cell count by colony counts incubated at 20°C for 3 days.

Results

Starvation

Changes in SH_{50} activity were regularly detected in juvenile salmonids starved under aquarium conditions at 10°C (Table 1). The activity in coho salmon and masu salmon starved for 10 days was 64% (relative % of the mean values) and 62% of that in fed animals, respectively. After 20 days the activity fell below limit of detection (2 units per ml) in coho salmon tested. Prolonged starvation followed almost all the salmon to an abnormal shape called pinhead.

Experimental Infection with Pathogenic Bacteria

The injection *i.p.* of viable cells (10^8) of *V. anguillarum* strain and *A. salmonicida* strain caused vibriosis and furunculosis in coho salmon and masu salmon, respectively (Table 2). In external observations the fish revealed little change until 1 day after injection. After 2 days the fish showed incipient signs of diseases (slight haemorrhage from anal and ventral and loss of appetite) and after 3 days, petechiae advanced in the vent and the fins. Almost all the fish became moribund after 5 days and the SH_{50} activity decreased to 30% for coho salmon injected with *V. anguillarum* and 35% for masu salmon injected with *A. salmonicida*. The bacteria inoculated were reisolated from the kidneys of all fish used.

Table 1. Effects of starvation on the SH_{50} activity of coho salmon and masu salmon sera

Fish and rearing	SH_{50}	Relative %
Coho salmon fed daily		
No. 1	15.5	
2	16.2	
3	16.9	
(Mean $\pm$ SD)	16.2 ± 0.7	100
Coho salmon starved for 10 days		
No. 1	8.5	
2	11.0	
3	11.7	
(Mean $\pm$ SD)	10.4 ± 1.7	64
Coho salmon starved for 20 days		
No. 1	<2	
2	<2	
3	<2	
(Mean $\pm$ SD)	<2	<12
Masu salmon fed daily		
No. 1	18.5	
2	19.2	
3	19.7	
(Mean $\pm$ SD)	19.1 ± 0.6	100
Masu salmon starved for 10 days		
No. 1	9.8	
2	12.5	
3	13.4	
(Mean $\pm$ SD)	11.9 ± 1.9	62

Effects of Viability of Pathogen

When coho salmon received *i.p.* 10^8 viable cells and 0.1 mg formalin-killed cells of *A. salmonicida* strain, a difference in the SH_{50} activity after 6 days appeared (Table 3). The viable cells decreased SH_{50} activity to 31% of that in uninjected fish and fell into a moribund condition, whereas the formalin-killed cells in which 0.1 mg wet cells corresponded to approximately 10^7 cells lost an ability to decrease the activity and to affect fish. The decrease in SH_{50} activity resulted from infection by the bacterium but not from the toxicity of cell components.

Naturally Diseased Fish

In naturally diseased coho salmon and masu salmon which survived in rearing condition and which were carried in the laboratory, sera were isolated and assayed for SH_{50} activity (Table 4). Coho salmon showed lesions in the epidermis where skin mucus disappeared but where no haemorrhage was seen. *Flexibacter* spp. were detected from smears of the lesions. The SH_{50} activity in the diseased fish fell into a level of 57% of that in the fish in which the bacteria were not detected.

Table 2. Changes in the SH₅₀ activity of coho salmon and masu salmon sera after the intraperitoneal injection of pathogenic bacteria, *Vibrio anguillarum* and *Aeromonas salmonicida*

Fish	Injected pathogen	Condition	SH ₅₀ * ¹	Relative %
Coho salmon				
No. 1	<i>V. anguillarum</i>	Moribund* ²	3.5	
2	<i>V. anguillarum</i>	Moribund* ²	4.1	
3	<i>V. anguillarum</i>	Moribund* ²	5.7	
4	<i>V. anguillarum</i>	Moribund* ²	6.4	
		(Mean±SD)	4.9±1.3	30
5	Not injected	Healthy	15.4	
6	Not injected	Healthy	16.1	
7	Not injected	Healthy	16.2	
8	Not injected	Healthy	17.0	
		(Mean±SD)	16.2±0.7	100
Masu salmon				
No. 1	<i>A. salmonicida</i>	Moribund* ²	4.4	
2	<i>A. salmonicida</i>	Moribund* ²	5.7	
3	<i>A. salmonicida</i>	Moribund* ²	6.6	
4	<i>A. salmonicida</i>	Unhealthy* ²	10.3	
		(Mean±SD)	6.8±2.5	35
5	Not injected	Healthy	18.5	
6	Not injected	Healthy	19.1	
7	Not injected	Healthy	19.4	
8	Not injected	Healthy	19.8	
		(Mean±SD)	19.2±0.6	100

*¹ The SH₅₀ activity was assayed 5 days after injection.*² The isolation of injected bacteria from the kidney succeeded.**Table 3.** Difference in the SH₅₀ activity of the serum of coho salmon injected with viable cells and formalin-killed cells of *Aeromonas salmonicida* intraperitoneally

Inoculum	N	SH ₅₀ * ¹		Relative %
		Range	Mean±SD	
Not injected	3	16.0–17.2	16.6±0.6	100
Viable cells (10 ⁸ per fish)	3	2.7– 7.5	5.2±2.4	31
Formalin-killed cells	3	15.4–16.6	16.0±0.6	96

*¹ The SH₅₀ activity was assayed 6 days after injection.

On the other hand, a great number of small gram-positive bacteria were detected from smears of the kidneys and livers of diseased masu salmon. The fish were affected with bacterial kidney disease (BKD). The SH₅₀ activity in diseased fish was 37% of that in unaffected fish. In a minute comparison between the externals and the SH₅₀ activity, the worst external signs of diseased individuals were closely related to the decrease in the SH₅₀ activity.

SH₅₀ Activity and Smoltification in Masu Salmon

There was a considerable difference in the SH₅₀ activity of masu salmon in parr-smolt transformation (Table 5). The SH₅₀ activity in smolt in which silvering in the epithelium appeared was 53% of

that in parr. Since the smolt showed no sign of disease and no bacteria was isolated from the kidneys, smoltification in masu salmon resulted in the decrease of SH₅₀ activity.

Discussion

A simple means would provide the fish farmer with a reliable tool for monitoring the health condition of fish. The stock enables prompt action to be taken when necessary at an early stage of developing health problems. A physiological parameter for such health assessment must satisfy the following five necessary conditions: (a) it must be sharply responsive to small changes of health condition, (b) it must be sensitive enough to detect

Table 4. The SH_{50} activity in the sera of naturally diseased salmonids

Fish	Detected pathogen	Condition	SH_{50}	Relative %
Coho salmon				
No. 1	<i>Flexibacter</i> spp.	Moribund	6.6	
2	<i>Flexibacter</i> spp.	Unhealthy	9.2	
3	<i>Flexibacter</i> spp.	Unhealthy	10.9	
		(Mean $\pm$ SD)	8.9 ± 2.2	57
4	Not detected	Healthy	13.8	
5	Not detected	Healthy	16.2	
6	Not detected	Healthy	16.5	
		(Mean $\pm$ SD)	15.5 ± 1.5	100
Masu salmon				
No. 1	BKD	Moribund	3.1	
2	BKD	Moribund	5.5	
3	BKD	Unhealthy	11.6	
		(Mean $\pm$ SD)	6.7 ± 4.4	37
4	Not detected	Healthy	15.9	
5	Not detected	Healthy	18.7	
6	Not detected	Healthy	19.4	
		(Mean $\pm$ SD)	18.0 ± 1.9	100

Table 5. The SH_{50} activity in parr-smolt transformation of masu salmon

Fish	SH_{50}	Relative %
Parr		
No. 1	18.5	
2	19.3	
3	20.0	
4	21.3	
(Mean $\pm$ SD)	19.8 ± 1.2	100
Smolt		
No. 1	9.9	
2	10.4	
3	10.5	
4	11.2	
(Mean $\pm$ SD)	10.5 ± 0.5	53

day by day changes but not hour by hour, (c) it must not vary in response to handling necessary for sampling, (d) the assay for a parameter must be brief and inexpensive, and (e) the analysis for data must be simple and time saving. Several parameters which involve whole blood analysis,^{1,9-12} plasma or serum analyses,^{2,13-18} and serum enzyme activities^{3,4} have been investigated in some fish species. Almost all the parameters which have been proposed for practical use, are considered to be not yet available because of the unsatisfaction at the foregoing necessary conditions.

It is well-known that normal fish sera possess a non-specific lytic activity against heterologous red blood cells, as reviewed by INGRAM.⁷ The haemolytic activity of fish sera is too powerful to compare with that of mammalian (goat, guinea

pig, rabbit and human) sera, as reported by SAKAI.⁸ At present, the interpretation of the mechanism of the non-specific haemolytic activity remains controversial and is divided into two opinions: one is activity dependent on the presence of naturally-occurring haemolytic antibodies and serum complement, and the other is considered non-antibody mediated implying complement activation in the absence of antibodies. The presence of an alternative pathway in the complement system of fish was claimed by NONAKA *et al.*¹⁷ for rainbow trout, GICLAS *et al.*¹⁸ for albacore tuna, and by OURTH and WILSON¹⁹ for channel catfish. Normal salmonid sera lyse GFRBC and the haemolytic activity has been expressed as SH_{50} .^{8,9} The present author interprets the SH_{50} activity in the serum as an alternative pathway activation, based on the response to chelating agents (EDTA and EGTA) and chemical activators.²⁰

The SH_{50} activity in fish serum was greatly changeable in response to the health condition of fish. When salmonids (coho salmon and masu salmon) were starved and injected with viable cells of *A. salmonicida* and *V. anguillarum*, changes in the SH_{50} activity were closely related to degree of starvation and infectious diseases, furunculosis and vibriosis (Tables 1 and 2). The decrease in the SH_{50} activity was traced by the daily assay of the activity: the SH_{50} activity decreased with time as the diseases progressed (data not shown). The decreasing responses of the SH_{50} activity to affected fish were correlated to deteriorating exter-

nal signs. Thus, a correlation between the decrease in SH₅₀ activity and the increase in severity of the starvation and diseases is evidently represented; that is, the SH₅₀ activity reflects the alteration in the state of fish health. In an attempt for the practical use of the SH₅₀ activity, a similar correlation was followed by naturally diseased fish (Table 4). Also, a considerable difference in the SH₅₀ activity was shown by parr-smolt transformation of masu salmon (Table 5). Hence, an assay of SH₅₀ activity bears a capacity to perceive not only the loss of a state of health but also slight physiological changes like smoltification which is a metamorphic phase into a sea-run type. The findings imply that the SH₅₀ activity is one of the more feasible parameters of fish health assessment. It is because the activity satisfies the five necessary conditions as a parameter. Therefore, it is considered possible that the assessment of the state of health is expressed in relative percents in each fish species by the application of an assay for the SH₅₀ activity.

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