Detection of *Vibrio anguillarum* from Wild Fingerlings of Ayu *Plecoglossus altivelis*

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From 1979 to 1983, detection of *Vibrio anguillarum* from wild fingerlings of ayu *Plecoglossus altivelis* were made by selective enrichment in alkaline peptone water and subsequent plating on BTB teepol agar. The detection rate was 0.08%, 2.5%, 6% and 17% from the fingerlings in Lake Biwa (freshwater lake), Lake Hamana (sea-water lake), the coastal sea of Hiwasa, and the estuary of the Hiwasa River, respectively. It was found that the fingerlings caught in sea water or blackish water harbored *V. anguillarum* at higher rates than those caught in the freshwater lake, and the pathogen was detected exclusively in the later period of the season for the fingerling collection in both freshwater and marine environments.

Among 54 isolates of *V. anguillarum*, 32 isolates were confirmed to be virulent for eels and most of the virulent strains belonged to the serotype J-O-1, J-O-2, or J-O-3. On the other hand, most of the avirulent strains could not be classified into any of the three serotypes.

Based on these results, it was deduced that *V. anguillarum* is introduced into freshwater ayu farms through the fingerlings themselves.

Ayu *Plecoglossus altivelis* (Salmonina: Plecoglossidae) is one of the most important species in freshwater fish culture in Japan, and recent annual production of farmed ayu exceeds 8,000 tons. Fry and fingerlings of this anadromous fish are caught as seedlings in estuaries along the southern coast of Japan or the coastal area of Lake Biwa, a freshwater lake where ayu are land-locked, from winter to spring. After a short period of acclimation, they are transferred to culture ponds supplied with clean running freshwater. There, they are raised up to the marketable size of 50-70 g for 3 to 4 months at water temperatures from 15°C to 25°C.

Vibriosis is the most serious disease problem in the ayu culture industry. It occurs in farms regardless of fish size, season, or source of seedlings, and the causative agent was confirmed as *Vibrio anguillarum* in a variety of cases.^{1,2)} Since 1973, there have been widespread outbreaks of the disease caused by drug resistant strains of the pathogen,³⁾ and this made the problem more serious.

Various methods of vaccination against the disease were confirmed effective by laboratory experiments,⁴⁻⁶⁾ and serological studies on the pathogen including isolates from other fish species were made.⁷⁻⁹⁾ However, few investigations have been made on vibriosis of ayu from an epizootiological point of view, and thus for example, the source of the infection has not yet been clarified. From the fact that the pathogen can not survive long in freshwater and the food used in ayu farms today is a commercial dry pellet, it seems most probable that the pathogen is brought into the culture ponds through the ayu themselves.

In this study, we tried to isolate *V. anguillarum* from fingerlings of ayu just after they were caught in natural waters or during the period in which they were kept temporarily in stocking net cages. As it was already known from experience that *V. anguillarum* could not be detected from such "carriers" by direct isolation onto agar plate media, an isolation procedure consisting of two steps with an enrichment liquid medium and a

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selective plate medium was employed following the method used for *V. cholerae* detection in the medical field.¹⁰⁾

Materials and Methods

Detection of *V. anguillarum*

From 1979 to 1983, several trials to isolate *V. anguillarum* from ayu fingerlings were made at Lake Biwa (the biggest freshwater lake in Japan, Shiga Prefecture), Lake Hamana (sea-water lake, Shizuoka Pref.), the sea shore of Hiwasa and the estuary of the Hiwasa River (Tokushima Pref.). Most of the fish used in this study, which were caught by fishermen using set nets, were apparently healthy though weakened or moribund fish were occasionally included.

The fish were swabbed on the body surface with alcohol-soaked cotton, incised aseptically, and put into alkaline peptone water (peptone 1%, NaCl 3%, pH 9.4). In some trials, specimens of a group were divided into two subgroups, and incised whole body samples were used in one subgroup. In the other subgroup, intestine, spleen, and liver were used as inoculation materials instead of the incised whole body. The difference in detection rate was, however, not significant between the two subgroups, and thus the results are presented in this paper without distinction to inoculation materials.

After incubation for 18 to 24 h at 25°C, one or two loopfuls of the liquid culture were streaked onto BTB teepol agar plates (Eiken) and incubated for another 48 h at the same temperature. Based on preliminary experiments, details such as the pH of the liquid medium, incubation temperature and period, and the kind of agar medium of the original method¹⁰⁾ were modified. The procedure employed in the present investigation is illustrated in Fig. 1.

Among yellow and transparent colonies grown on BTB teepol agar plates, those colonies resembling *V. anguillarum* were picked up and transferred to nutrient agar plates (Eiken, NaCl 0.5%). This selection was done empirically and/or by referring to some typical colonies of *V. anguillarum* strains. The same selection was made again for the colonies on the nutrient agar. These isolates selected as presumptive-*V. anguillarum* were submitted to glucose utilization, vibrio-static agent O/129 sensitivity, and other detailed characterization tests. In the cases that many isolates of presumptive-*V. anguillarum* were obtained

Fig. 1. Method for detecting *Vibrio anguillarum* from fingerlings of ayu.

from the same group of fish, some representative isolates were submitted to the tests. Finally, serological and pathological tests were done on the isolates which were identified as *V. anguillarum*.

Serological Test (Agglutination)

Slide agglutination tests were carried out on 69 strains of *V. anguillarum* with rabbit anti-*V. anguillarum* sera against O-antigens of three serotypes, J-O-1, J-O-2, and J-O-3⁷⁾ (each type corresponds to A, B, and C, respectively, designated by KITAO *et al.*⁹⁾). The sera were presented by Dr. KITAO or prepared in our laboratory at Hiroshima University.

Pathological Test

Pathogenicity of 54 isolates of *V. anguillarum* was tested by using Japanese eel (*Anguilla japonica*), which was chosen as the experimental animal for convenience and based on the fact that almost all of the *V. anguillarum* strains isolated from diseased ayu showed pathogenicity for the eel.¹⁾

Each 24 h-culture of the 54 isolates on nutrient agar at 25°C was suspended in sterilized saline (NaCl 0.85%) and injected intramuscularly to two eels with a dose of 1 mg of cells per 100 g of fish body weight. The injected fish were kept in freshwater tanks at about 20°C and mortalities were recorded for 7 or 10 days. The strain which killed either one or both of the injected fish within the period was interpreted as virulent.

Results

Detection of *V. anguillarum*

The result of detection of *V. anguillarum* from ayu fingerlings caught in Lake Biwa from 1979 to 1981 is given in Table 1. As shown in the table, *V. anguillarum* was isolated from only 4 fish out of 5044 fish examined, and these 4 fish were all caught in June.

Table 1. Detection of *V. anguillarum* from fingerlings of ayu caught in Lake Biwa (freshwater lake) from 1979 to 1981

	Number of fish from which <i>V. anguillarum</i> was isolated			Number of fish examined
	April	May	June	Total
1979	0/847	0/1077	1/720	1/2644
1980	0/400	0/400	3/400	3/1200
1981	0/400	0/400	0/400	0/1200
Total	0/1647	0/1877	4/1520	4/5044
Rate	0%	0%	0.26%	0.08%

Mean body weight of fish examined and water temperature.			
	April	May	June
1979	2.2g (14.3°C)	2.3g (14.7°C)	2.7g (18.0°C)
1980	1.6 (12.0)	1.1 (13.4)	3.8 (17.8)
1981	1.0 (10.9)	2.2 (14.7)	3.4 (15.5)

The result of detection made in Lake Hamana is shown in Table 2. The rate of detection through the four years was 0% in December and January, 1.1% in February, and 7.4% in March, and the overall rate obtained in this sea-water lake was 2.5%.

Table 3 shows the results of isolation trials made in Hiwasa. The detection rate was 6% in the samples caught in the sea and 17% in the samples from the river.

During and after the characterization tests, a substantial percentage of the presumptive-*V. anguillarum* strains were proven not to be *V. anguillarum*. Table 4 gives the biochemical characteristics of 69 isolates which were finally identified as *V. anguillarum*. These characteristics are consistent with those of *V. anguillarum* isolated from ayu affected by vibriosis in culture ponds,¹⁾ with the exception of a negative reaction in arginine decomposition of 2 strains from Lake Hamana, 1982.

Serological Test

Among 69 isolates tested, 8 (11.6%), 12 (17.4%), and 23 (33.3%) isolates were classified to the serotype J-O-1, J-O-2, and J-O-3, respectively, and the remaining 25 (37.7%) isolates did not agglutinate with any of the three types of antisera (Table 5).

Pathological Test

Among 54 isolates tested, 32 (59%) isolates were found to be virulent (Table 6).

Discussion

It was confirmed by the present investigation

Table 2. Detection of *V. anguillarum* from fingerlings of ayu caught in Lake Hamana (sea-water lake) from 1980 to 1983

	Number of fish from which <i>V. anguillarum</i> was isolated / Number of fish examined				
	Jan.	Feb.	Mar.	Dec.	Total
1980				0/480	0/480
1981		4/550	41/400	0/400	45/1350
1982	0/400	8/400	32/400		40/1200
1983		1/200	1/200		2/400
Total	0/400	13/1150	74/1000	0/800	87/3430
Rate	0%	1.1%	7.4%	0%	2.5%

Mean body weight of fish examined and water temperature.				
	Jan.	Feb.	Mar.	Dec.
1980				0.07g (15.9°C)
1981		0.70 (11.3)	1.80 (12.6)	0.17 (15.0)
1982	0.18 (8.5)	0.60 (8.9)	0.90 (19.7*)	
1983		0.79 (9.8)	2.15 (16.4*)	

* water temperature of stocking ponds.

Table 3. Detection of *V. anguillarum* from fingerlings of ayu caught in the coastal sea of Hiwasa and the estuary of the Hiwasa River in 1980

		Number of fish from which <i>V. anguillarum</i> was isolated	Number of fish examined
1980	March	Coastal sea	60/1000 (6%)
	May	Hiwasa River	17/100 (17%)

Mean body weight of fish examined and water temperature.

coastal sea: 0.8 g (15.0°C)

Hiwasa River: 1.6 g (17.2°C), chlorinity 4‰.

that some fingerlings of ayu harbored *V. anguillarum* at the time when they were captured in natural waters, and these carriers or infected seedlings are thought to be the source of infection in farms. It could not be clarified whether these *V. anguillarum*-harboring fingerlings were in carrier phase or infected phase, since the health condition of the fish was not checked individually.

A variation in detection rate from place to place was demonstrated. The rate obtained in Lake Biwa, a freshwater lake, was 0.08%,

and it was much lower than those obtained in the sea-water lake (Lake Hamana, 2.5%), the sea shore (Hiwasa, 6%), and the estuary (Hiwasa River, 17%). From these results, it can be said that marine-seedlings of ayu harbor *V. anguillarum* at a higher rate than freshwater-seedlings.

The detection rate also varied considerably with the time of sample collection. In Lake Biwa, *V. anguillarum* was isolated only in June, almost the end of the season for seedling collection in the lake. The same phenomenon was observed also in Lake Hamana. *V. anguillarum* was detected exclusively from the fish caught in February and March, the latter half of the season for fingerling collection in this sea-water lake. Though this difference in detection rate is thought to be influenced by water temperature, the critical temperature could not be drawn from the present data. In any case, it is advisable that ayu culturists avoid introducing those fingerlings caught in the later season from either Lake Biwa or sea waters.

Table 4. Biochemical characteristics of *V. anguillarum* strains isolated from fingerlings of ayu

Test	Strains										
	Lake Biwa (4 strains)	Hiwasa River (11 strains)	Hiwasa, sea (15 strains)	Lake Hamana 1981 (13 strains)	L. Hamana 1982 (26 strains)		Lake Biwa	Hiwasa River	Hiwasa, sea	L. Hamana, 1981	L. Hamana, 1982
OF test	F	F	F	F	F	glucose	+	+	+	+	+
Gas from glucose	—	—	—	—	—	mannose	+	+	+	+	+
Oxidase	+	+	+	+	+	maltose	+	+	+	+	+
Sensitivity to O/129	—3*	+	+	+	+	trehalose	+	+	+	+12	—16
novobiocin	+	+	+	+	+	dextrin	+	+	+10	+	+
Litmus milk	+	+	+	+	+	mannitol	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	starch	+	+	+	+	+
Gelatin liquefact.	+	+	+	+	+	sucrose	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	glycogen	+	+	+	+	+
Indole	+	+	+	+	+	glycerin	—3	—9	+	—	+
Methyl red	—	—10	—10	—	—	cellobiose	—3	+	+	+10	+
Voges-Proskauer	+	+	+	+	+	arabinose	—	+8	+	+	+
2,3-butanediol prod.	+	+	+	+11	+	inositol	—	—10	—	—	—
Hydrogen sulfide	—	—	—	—	—	sorbitol	+	+10	+	—	+
Arginine decomp.	+	+	+	+	+24	lactose	—	—	—	—	—
Lysine decarboxyl.	—	—	—	—	—	inulin	—	—	—	—	—
Ornithine	—	—	—	—	—	rhamnose	—	—	—	—	—
Urease	—	—	—	—	—	xylose	—	—	—	—	—
Citrate (Simmons)	+	+	+	+	+	raffinose	+3	—	—	—	—
Acid from						adonitol	—	—	—	—	—
fructose	+	+	+	+	+						
galactose	—3	+	+	+11	+						

* number of strains showing reaction as given.

Table 5. Serotype of *V. anguillarum* strains isolated from fingerlings of ayu

Source of strains	Number of strains examined	J-O-1 (A)	J-O-2 (B)	Serotype J-O-3 (C)	Others
Lake Biwa (1979, 1980)	4	3	0	0	1
Hiwasa River (1980)	11	1	1	0	9
Hiwasa, sea (1980)	15	0	2	6	7
Lake Hamana (1981)	13	4	0	1	8
Lake Hamana (1982)	26	0	9	16	1
Total	69	8 (11.6%)	12 (17.4%)	23 (33.3%)	26 (37.7%)

Table 6. Pathogenicity for Japanese eel of *V. anguillarum* strains from fingerlings of ayu

Source of strains	Number of strains examined	Number of virulent* strains (%)
Lake Biwa	4	3 (75)
Hiwasa, River	10	4 (40)
Hiwasa, sea	14	7 (50)
Lake Hamana, 1981	13	6 (46)
Lake Hamana, 1982	13	12 (92)
Total	54	32 (59%)

* Strains which killed eels (*Anguilla japonica*) by intramuscular injection with 1 mg/100 g fish body weight were interpreted as virulent.

Serotyping of *V. anguillarum* strains isolated from diseased ayu were made by several workers. EZURA *et al.* (1979)⁷⁾ reported that 99, 10, and 14 strains among 124 strains tested belonged to J-O-1, J-O-2, and J-O-3, respectively, and only one strain did not belong to any of the three types. KUSUDA *et al.* (1981)⁸⁾ and Jo²⁾ (1981) noted that all the isolates from diseased ayu could be classified to one of these three types. KITAO *et al.* (1983)⁹⁾ demonstrated some minor serotypes in addition

to these three types, however, they also classified most of the ayu isolates to the three serotypes. In the present study, about one third of the isolates (26/69) could not be classified to any of the three types. At the same time, it was revealed that about 40 percent of the isolates were not virulent for the eel and thus the relationship between serotype and pathogenicity was examined by combining the two data, as shown in Table 7. As presented in the table, only 4 strains out of 25 strains of unknown serotypes were virulent, while 28 out of 29 strains belonging to one of the three types were virulent. This means that the strains of *V. anguillarum* isolated from fingerlings of ayu are not always virulent ones and most of the virulent strains are included in the three serotypes, J-O-1, J-O-2 or J-O-3. From this result and the above mentioned fact that the three serotypes were exclusively isolated from diseased ayu in farms, it is concluded that the procedure shown in Fig. 1 can be simplified by introducing serological test with antisera against the three types of *V. anguillarum* in place of the characterization tests, though the minority of virulent strains

Table 7. Relationship between pathogenicity and serotype of *V. anguillarum* isolated from fingerlings of ayu

Number of strains examined	54	Virulent	32	Serotype	
				J-O-1	8
				J-O-2	10
				J-O-3	10
		Others		4	
		Avirulent	22	J-O-1	0
				J-O-2	0
				J-O-3	1
				Others	
		<hr/>			
		Serotype	Number of strains virulent/tested		
		J-O-1	8/8 (100%)	} 28/29 (97%)	
		J-O-2	10/10 (100%)		
		J-O-3	10/11 (91%)		
		Others	4/25 (16%)		

belonging to the other serotypes will escape detection.

The three serotypes, J-O-1, J-O-2, and J-O-3 were interpreted as freshwater type, intermediate type, and marine type, respectively.⁸⁾ However, as demonstrated in the present study, J-O-1 type strains were isolated from marine-seedlings caught in Lake Hamana and the estuary of the Hiwasa River and thus at present, the above mentioned ecological demarcation of the three serotypes is not always valid. Serotype J-O-1 comprised the majority of the strains isolated from diseased ayu in farms,⁷⁻⁹⁾ but it was not major type among the strains isolated from fingerlings. Therefore, J-O-1 type seems to be the most virulent type among the three virulent types, and this interesting point is left for a further investigation.

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