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Vibriosis of Ayu : A Review

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INTRODUCTION

Ayu, *Plecoglossus altivelis* (Salmoniforms: Plecoglossidae), is one of the most important species among cultured freshwater fishes in Japan, and the recent annual production of farmed ayu reaches 10,000 tons. The wild populations of this anadromous fish spend most of their one year life in freshwater environments. After spending their first few months in the sea or in Lake Biwa where land-locked stocks of ayu inhabit, juveniles or young fish enter rivers in the spring and gradually migrate upstream. By the late summer they begin to migrate back to the lower reaches of the river, where they spawn in the fall. The newly hatched larvae are carried to the sea or the lake. The juveniles of ayu are caught in estuaries along the south-coast Japan or the coastal area of Lake Biwa through the late winter into the early spring, and transferred to freshwater ponds for culture, or released into rivers for fisheries stock and game fishing. Recently, a considerable amount of seedlings raised from artificially inseminated eggs has come to be supplied at many hatcheries.

In culture ponds, ayu are raised up to a marketable size of 30-100 g for 2 to 4 months at water temperatures from 15 to 25°C (optimum range: 22-24°C). Frozen trash fishes were given to ayu in the past, but nowadays pelleted or crumbled artificial diets are generally used.

No viral disease has been reported until quite recently, however, a mortality due to IHNV (Infectious haematopoietic necrosis virus)-like virus was observed in 1986 (UGAJIN, 1987; YOSHIMIZU

et al., 1987)*1, and a rhabdovirus (HRV) pathogenic to Japanese flounder (*Paralichthys olivaceus*) was isolated from larval ayu (Kimura *et al.*, 1986)[1]. On the other hand, various bacterial diseases have been recorded in ayu. *Vibrio anguillarum* (Muroga and Egusa, 1967)[2], *V. ordalii* (Muroga *et al.*, 1986)[3], *V. cholerae* non-O-1 (Muroga *et al.*, 1979)[4], *Vibrio* sp. (Kusuda, 1965)[5], *Aeromonas hydrophila* (Jo and Ohnishi, 1980)[6], *A. salmonicida* (unpublished), *Pseudomonas anguilliseptica* (Nakai *et al.*, 1985)[7], *Flavobacterium* sp. (Miyazaki and Jo, 1986)[8], *Pasteurella plecoglosacida* (Kusuda and Miura, 1972)[9] and *Streptoccocus* sp. (Kitao *et al.*, 1981; Ohnishi and Jo, 1981; Ugajin, 1981)[10-12] have been reported as disease agents from farmed ayu. Among them, *V. anguillarum* is beyond comparison the most devastating pathogen of this fish.

Two decades have passed since *V. anguillarum* was first reported as a disease agent in ayu, and during this period various investigations were made especially on the immunological prophylaxis. As a result, commercial vaccines are expected to become available for ayu vibriosis in the near future. Under the circumstances, it seems worthwhile to review the past achievements on the vibriosis of ayu at this stage. In this context, a number of pages is assigned to the studies on vaccination in this review.

CAUSATIVE AGENT : *Vibrio anguillarum*

In the early 1960's, vibriosis was already known as the most serious disease in ayu culture (Ishida, 1964)*2, even though the causative bacterium was not formally identified. This seems to be based on a brief description made by Hoshina that ayu was attacked by a vibrio (*V. piscium* var. *japonicus*), the causative agent of vibriosis in rainbow trout (Hoshina, 1956, 1957)[13,14].

In 1967, the causative agent of the vibriosis occurring in juvenile and young ayu caught in Lake Hamana (sea-water lake) was identified as *V. anguillarum* (Muroga and Egusa)[2]. Successively, the same pathogen was isolated from diseased ayu juveniles caught in another district (Muroga and Motonobu, 1967)[15] and farmed ayu in freshwater ponds (Muroga and Egusa, 1970)[16]. From then on, the bacterium has been isolated from ayu in various districts of Japan (Kodera *et al.*, 1974; Uno and Muroga, 1974; Kusuda *et al.*, 1979; Ezura *et al.*, 1980)[17-20].

General characteristics

Several studies on morphological, biochemical, physiological and genetical characteristics of *V. anguillarum* isolated from ayu and other fishes have been carried out (Egusa, 1969; Muroga and Egusa, 1973; Muroga, 1975; Kusuda *et al.*, 1979; Muroga *et al.*, 1984; Tajima *et al.*, 1985, 1986)[21-23,19,24-26]. Until the first half of the 1970's, there was some confusion about the definition of *V. anguillarum* (Egusa, 1969)[21], but now it is well defined as listed in Bergey's Manual (Krieg and Holt, 1984)[27]. One remaining problem is that the bacterium proposed as *Vibrio ordalii* by Schiewe *et al.* (1981)[28] should be included in *V. anguillarum* as biotype II as shown in the Bergey's Manual, or should be separated. In this review, *V. ordalii* is excluded from *V. anguillarum* to avoid confusion.

Several important characteristics of *V. anguillarum* are listed in Table 1 according to the

*1 Ugajin, M.: A viral disease occurring in cultured ayu.; Yoshimizu, M. *et al.* : Characteristics of a virus isolated from diseased ayu. Both in Abstract of Annual Meeting of the Japanese Society of Fish Pathology (Showa 62, April) pp. 11-12 (1987). (in Japanese)

*2 Ishida, R.: Vibriosis of ayu, *Yohshoku*, 1(6), 44-46 (1964). (in Japanese)

Table 1. Characteristics of *Vibrio anguillarum* isolated from ayu

Character	Bergey's Manual (1984)[27]	Muroga & Egusa (1967)[2] 6 strains	Wakabayashi *et al.* (1975)[29] 4 strains	Muroga *et al.* (1979)[30] 52 strains
Motility	+	+	−	+51*1 − 1
Sensitivity to 0/129	+	+	+	+25 −27
Nitrate reduction	+	+	+	+49 − 3
Gelatinase	+	+	+	+
Gas from glucose	−	−	−	−
Arginine decomposition	+	+	+	+
Lysine	−	−*2	−	−
Ornithine	−	−	−	−
MR	−	−	−	+ 4 −48
VP	+	+	+	+
Acid from				
glucose	+	+	+	+
sucrose	+	+	+	+
cellobiose	+	+5 −1		+13 −39
mannitol	+	+	+W	+42 −10
arabinose	+	+3 −3	−	+23 −29
NaCl				
0%	d*3	−	−	−
5			+1 −3	+29 −23
6	d			
7		+	−	−
8	−	−		

*1: number of strains having reactions as given.

*2: tested later by the present authors.

*3: 11-89% of strains are positive.

+W: weakly positive.

description of the Bergey's Manual. In addition to this, the characteristics of the first reported strains (Muroga and Egusa, 1967)[2] and some important exceptional strains are shown in the table. Non-motile *V. anguillarum* strains were isolated from diseased ayu in Nagano (Wakabayashi *et al.*, 1975)[29] and Tokushima (Muroga *et al.*, 1979)[30] Prefectures. Sensitivity to vibrio-static agent 0/129 (pteridine compound) is one of the important requisites for the identification of the genus *Vibrio,* however, especially in 1976 and 1977, *V. anguillarum* strains non-sensitive to 0/129 were frequently isolated from affected ayu (Muroga *et al.*, 1979)[30]. At that time a sulfadoxine/trimethoprim (ST) mixture was used illegally by a number of culturists, and it was confirmed by an in vitro experiment that strains sensitive to both 0/129 and ST mixture became resistant not only to ST mixture but also to 0/129 by repeated exposures to low concentration of ST mixture (Muroga *et al.*, 1979).

Pathogenicity

Strains of *V. anguillarum* isolated from diseased ayu were pathogenic not only to ayu itself but also to other fishes such as Japanese eel (*Anguilla japonica*), European eel (*A. anguilla*), loach (*Misgurnus anguillicaudatus*), goldfish (*Carassius auratus*), crucian carp (*C. carassius*), Amago (*Oncorhynchus rhodurus* f. *macrostomus*), kokanee salmon (*O. nerka* f. *adonis*), black seabream (*Acan-*

thopagrus schlegeli), and yellowtail (*Seriola quinqueradiata*) (Muroga, 1975; Uno, 1976)[23,31]. Among these fish species, ayu is thought to be the most susceptible fish; the lethal dose of a highly virulent strain in intramuscular (IM) and intraperitoneal (IP) injections in this fish was reported c. a. 8 cells and 17 cells per fish, respectively, and the lethal concentration in water-born infection was 10^4 cells/mℓ (Jo and Muroga, 1977; Jo, 1981; Kusuda *et al.*, 1981; Aoki *et al.*, 1984)[32-35]. Intragastric inoculation with 6.3×10^5 cells/fish could also kill ayu (Kusuda *et al.*, 1981; Minami *et al.*, 1983)[34,36], whereas Kodera *et al.* (1974)[37] reported that oral administraion (3 mg/fish) of a strain could not introduce the infection in ayu. As mentioned later, three serotypes of *V. anguillarum* have been isolated from diseased ayu. Among them, J-O-1 (A) seems to be the most virulent type (Minami *et al.*, 1983)[36], however, the precise relationship between serotype and virulence is yet to be investigated. Mouse was not susceptible to *V. anguillarum* strains (Muroga, 1975)[23].

Various virulence factors of *V. anguillarum* have been enumerated (Evelyn, 1984)[38], however, the importance of each factor has not been fully elucidated. As for the strains from ayu, an extracellular protease was demonstrated as a lethal factor for ayu and other fishes (Inamura *et al.*, 1984, 1985)[39,40].

Serotype

O-serotyping of Japanese strains of *V. anguillarum* has been carried out independently in three laboratories, Miyazaki University (Aoki and Kitao, 1978; Kitao *et al.*, 1983, 1984)[41-43], Hokkaido University (Ezura *et al.*, 1980; Tajima *et al.*, 1985, 1986)[20,25,44,45] and Kochi University (Kusuda *et al.*, 1981)[46]. They, though using different designations for typing, agreed with each other that most strains of *V. anguillarum* isolated from diseased fishes in Japan could be divided into three serotypes. In this paper, the designation proposed by Ezura *et al.* (1980)[20] is adopted. In Table 2, results of O-serotyping of *V. anguillarum* strains from diseased ayu reported by the above-mentioned three groups of researchers and Jo (1981)[33] are collected. As shown there, 86% of the examined strains fell into J-O-1, and only 5% and 8% of the strains belonged to J-O-2 and J-O-3, respectively. In addition to the serotyping, comparative analyses of proteins and lipopolysaccharides (LPS) among the representative serotypes have been carried out (Aoki *et al.*, 1981; Salati and Kusuda, 1986)[47,48].

While J-O-1 type was isolated from diseased ayu in freshwater ponds regardless of their history of early life, J-O-2 and J-O-3, especially the latter which was called marine type (Kusuda *et al.*, 1981)[46], were usually isolated from juvenile or young ayu caught in marine environments during the acclimation period from sea water to freshwater or just after the acclimation (Jo, 1981; Mifuchi *et al.*, 1983)[33,49]. Mifuchi *et al.* (1983)[50] investigated the relationship between serotype and drug sensitivity of the isolates from diseased ayu in Lake Hamana, and they found

Table 2. Serotyping of *V. anguillarum* strains isolated from diseased ayu

Serotype	Ezura *et al.* (1980)[20]	Kusuda *et al.* (1981)[46]	Jo (1981)[33]	Kitao *et al.* (1983)[42]	Mean (%)
J-O-1	99* (80%)	13 (87)	215 (84)	241 (92)	86
J-O-2	10 (8)	0 (0)	17 (7)	6 (2)	5
J-O-3	14 (11)	2 (13)	24 (9)	12 (5)	8
Others	1 (1)	0 (0)	0 (0)	4 (2)	1

* number of strains.

an interesting fact that J-O-1 strains were resistant to nalidixic acid, oxolinic acid and nifulpirinol, while J-O-2 and J-O-3 strains were sensitive to these drugs. They noted that the replacement of the causative agent from J-O-2 and J-O-3 serotypes to J-O-1 serotype along with proceeding of acclimation and rearing could be attributed to a selection by certain chemotherapeutics used in these periods.

Recently, O-serotyping scheme of *V. anguillarum* was reported from Denmark (SKOV SØRENSEN and LARSEN, 1986)[51]. In their scheme, 10 serotypes were listed and J-O-1, J-O-2 and J-O-3 correspond to Denmark O2, O3 and O1, respectively. It has been revealed that there were several minor serotypes of Japanese strains (KITAO *et al.*, 1984; TAJIMA *et al.*, 1986)[43,45]. Furthermore, many O-serologically untypable strains have been isolated from wild fingerlings (MUROGA *et al.*, 1984)[52], reared seedlings of ayu (TATANI *et al.*, 1985)[53] and sea waters (MUROGA *et al.*, 1986)[54]. Most of these untypable strains isolated from healthy fish or environments were non-pathogenic, however, a disease case due to an untypable strain of *V. anguillarum* occurred in ayu fingerlings under seed production (TATANI *et al.*, 1985)[53].

There have been very few investigations on thermo-labile antigens of *V. anguillarum*. TAJIMA *et al.* (1985)[25] reported that the representative strains of *V. anguillarum* (J-O-1～J-O-8) have a common heat-labile antigen (k-1) and *V. ordalii* strains have another specific antigen (k-2). Recently, RASMUSSEN (1987)[55] reported that 11 strains of Denmark O1 *V. anguillarum* including a Japanese strain PT-213 (J-O-3) have a common K antigen (K-1).

EPIDEMIOLOGY

V. anguillarum infection in ayu occurs in any stages of fish development both in freshwater and sea water environments throughout the year. There have been some papers describing the occurrence of vibriosis in juveniles of ayu in seed production process (MUROGA *et al.*, 1974; TAKEDA *et al.*, 1975; TATANI *et al.*, 1985; IWATA *et al.*, 1988)[56,57,53,58]. It was revealed that rotifer (*Brachionus plicatilis*), a main diet for ayu larvae, played the role of vector of the pathogen (TABATA *et al.*, 1982; TATANI *et al.*, 1985)[59,53]. TABATA *et al.* (1982) suggested that a bathing of rotifers in furanace, a nitrofuran compound used at that time, would reduce the mortality due to the vibriosis. However, as far as the current system of seed production is kept on, that is, ayu are reared receiving rotifers in sea water, it seems impossible to avoid vibriosis because *V. anguillarum* is ubiquitous in inshore waters at least in the Inland Sea of Japan and its adjacent areas (MUROGA *et al.*, 1986)[54].

The source of the infection in ayu occurring in freshwater ponds is thought to originate from carrier fish, because the pathogen cannot survive in freshwater environments (ITAMI and KUSUDA, 1984; MUROGA *et al.*, 1986)[60,54]. In fact, *V. anguillarum* was detected in seedlings caught in either marine or freshwater environments (MUROGA *et al.*, 1984)[52]. The carrier rate became higher towards the end of the season for seedling collection, thus the preference of the juveniles caught in the early months of the season will result in higher survival rate.

V. anguillarum is generally thought as a pathogen of marine fishes and migratory fishes like salmon and eels (ANDERSON and CONROY, 1970)[61]. However in Japan, as described repeatedly, *V. anguillarum* infection occurs frequently in farmed populations of ayu in freshwater ponds, and *V. anguillarum* was detected from juvenile ayu caught in Lake Biwa (MUROGA *et al.*, 1984)[52]. It is an interesting problem how the pathogen has remained and clung to ayu successively in this

freshwater lake, considering that the organism was revealed experimentally to be unable to survive in freshwaters (Itami and Kusuda, 1984; Muroga *et al.*, 1986)[60,54].

SYMPTOMS AND PATHOLOGY

In juvenile ayu, the symptom of the vibriosis is characterized by cloudy area in the trunk (Muroga and Egusa, 1967)[2]. In young and adult stages, spots or lines of hemorrhages are observed on the trunk skin. Other symptoms like exophthalmus, reddening and expansion of anus, and ulcerative lesions of the skin are sometimes observed. Intestinal hemorrhage and congestion of the liver, spleen and kidneys are seen in affected fish especially at progressed stages (Funahashi *et al.*, 1974; Egusa *et al.*, 1979; Kubota *et al.*, 1982)[62-64]. The pathological changes in the blood have been scarecely studied owing to the difficulty in obtaining an enough amount of blood sample from this small fish.

The speed of progress of the infection is influenced by water temperature, but it usually requires only 2 to 4 days from the onset of the infection to individual death (Jo, 1981)[33].

The gills, skin and gastrointestinal tract have been implicated as the site of entry of *V. anguillarum* in salmonid fishes (Evelyn, 1984)[38]. In ayu, Funahashi *et al.* (1974)[62] observed that the first focal infection occurred in the skin of naturally and experimentally infected fish. Kawai *et al.* (1981)[65] and Muroga and De La Cruz (1987)[66] concluded that the skin was the site of entry for the pathogen based on the experimental infections, though the possibility that the pathogen enters through the gills and intestinal tract cannot be totally ruled out. After the proliferation in the first colonization site, the bacteria are distributed to various parts of the body and finally cause septicemia (Funahashi *et al.*, 1974; Egusa *et al.*, 1979; Muroga and De La Cruz, 1987)[62,63,66].

Recently, a lytic activity against *V. anguillarum* cells was demonstrated in the skin mucus of ayu (Itami *et al.*, 1987)[67] and a virulence-enhancing effect of injected iron compounds was confirmed in experimental ayu vibriosis (Nakai *et al.*, 1987)[68], however, the various factors influencing the host-parasite relationship in ayu vibriosis remain to be investigated.

CONTROL MEASURES

Since nitrofuran derivatives, sulfa drugs and antibiotics had been reported to be effective for the control of vibriosis in rainbow trout (Hoshina *et al.*, 1957; Saito *et al.*, 1964; Hayashi *et al.*, 1964, 1965)[69-73], these chemotherapeutics were also used in ayu farms in the 1960's. Later, bathings in sulfamonomethoxine (Ogami, 1969)[74] and chlortetracycline (Muroga and Egusa, 1968)[75] were confirmed to be effective against vibriosis occurring in seedling ayu caught in the sea. In those days vibriosis was not a serious threat for ayu farmers because it was easily controlled by chemotherapy. Almost suddenly in 1973, the vibriosis prevailed in various districts of Japan due to the ineffectiveness of sulfa drugs and antibiotics, and the economic loss reached 670 million yen in that year. Since then, vibriosis has become the most important obstacle to ayu culture, and studies on immunological prophylaxis have been taken up.

Chemotherapy

In a guide-book for ayu culture (Ogami, 1966)*3, oral administration of sulfa drugs (sulfisox-

*3 Ogami, H.: Ayu—Techniques for pond culture—. Nohsangyoson-Bunka-Kyokai, Tokyo, pp. 1-115 (1966). (in Japanese)

asole, sulfathiazole, sulfadimethoxine, sulfamonomethoxine), nitrofurans (furazolidone), and antibiotics (tetracycline) were recommended as control measures for vibriosis. In fact, most of the strains isolated from diseased ayu in the 1960's were sensitive to at least tetracycline and chloramphenicol, which were often used as sovereign remedies in ayu farms. Nevertheless, as mentioned above, the isolates from infected ayu in 1973 were resistant to sulfamonomethoxine, tetracycline and chloramphenicol (Jo, 1981)[33]. Aoki *et al.* (1974)[76] exhibited that 65 out of 68 strains isolated from epidemics of vibriosis in 1973 carried transferable drug resistance factor (R factor). The most common R factor encoded with resistance to four drugs (sulfonamides, streptomycin, chloramphenicol and tetracycline). They concluded that the selective pressure exerted by chemotherapeutic agents used in fish farms caused the high incidence of R factor in this pathogen. Thereafter, studies on the occurrence of resistant strains and the effects of chemotherapeutics on bacterial ecology in ayu culture environments have been continued by them (Aoki, 1975; Aoki *et al.*, 1980, 1981, 1984, 1985)[77-81]. The changes in drug resistance pattern and carring rate of R-plasmid in *V. anguillarum* isolated from diseased fish (mainly from

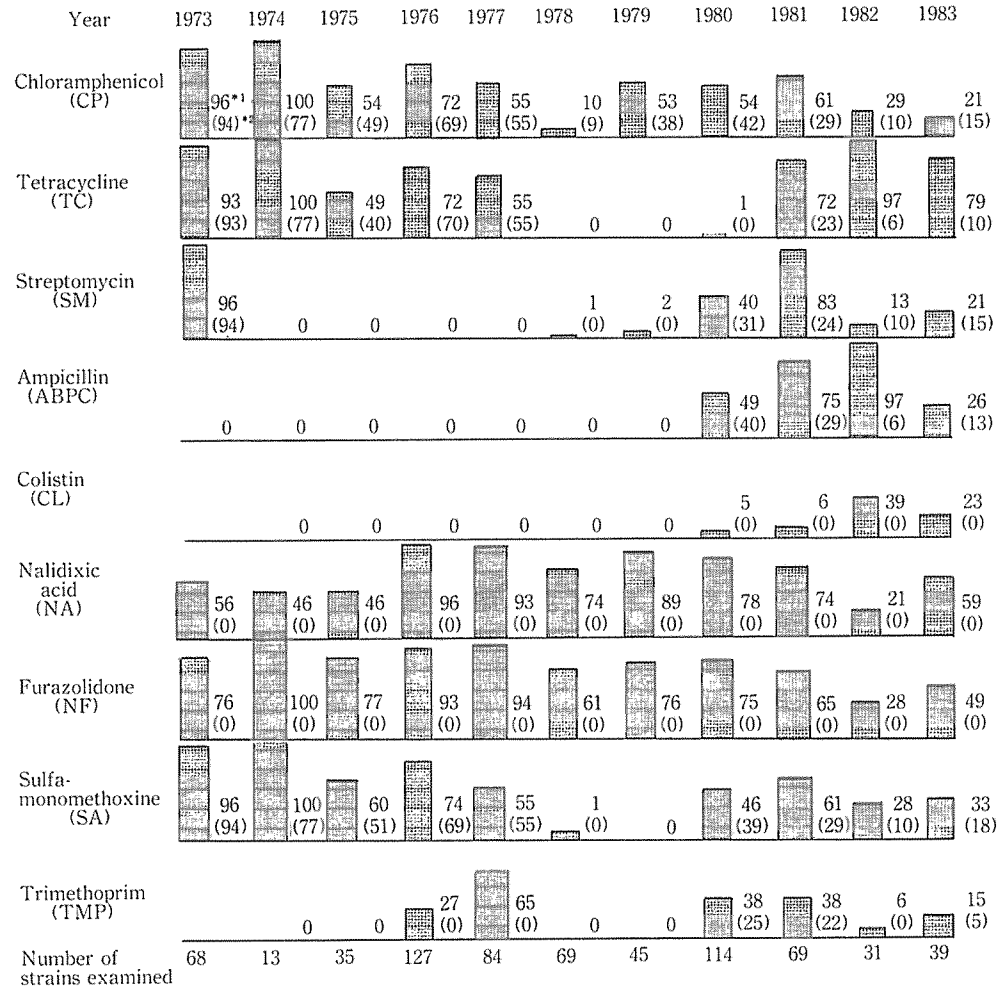

Fig. 1. Changes in drug resistance and R-plasmid of *Vibrio anguillarum* from ayu. (from Aoki *et al.*, 1985)[81] *1 Percentage of drug resistant strains *2 Percentage of R^+ strains

ayu) from 1973 to 1983 are shown in Fig. 1.

There have been very few papers reporting the practical efficacy of medication in ayu farms, however, Kusuda *et al.* (1981)[82] described an interesting case in which the treatment for vibriosis with oxolinic acid and oxytetracycline often resulted in occurrence of another disease (*Streptococcus* infection).

In Japan, the legal restriction for medication in fish culture was enforced in 1981, and oxytetracycline, chlortetracycline, sulfamonomethoxine and furazolidone were allowed to use for ayu vibriosis. Later, the tetracyclines and furazolidone were omitted from the list, and at present oxolinic acid, nalidixic acid, sulfamonomethoxine, a mixture of sulfamonomethoxine and ormethoprim, sulfisoxasole and colistin sulfate are permitted to use for the control of ayu vibriosis (1988, Fisheries Agency, Japanese Government).

Vaccination

Studies on vaccination against vibriosis in rainbow trout were commenced in as early as the 1960's in Japan (Hayashi *et al.*, 1964; Saito *et al.*, 1964; Hoshina *et al.*, 1965)[72,70,83]. Independently of these studies the investigation on immunological prophylaxis against ayu vibriosis was started around 1975 under the stimulus of the following two events: one, as a matter of course, was frequent occurrence of ayu vibriosis due to the appearance of drug resistant strains, and the other was the success of oral vaccination against salmon vibriosis in the USA (Fryer *et al.*, 1972, 1976; Rohovec *et al.*, 1975)[84-86].

The first successful result of oral vaccination was reported by Kusuda *et al.* (1978)[87], and successively, following the developments of new vaccination methods in the USA (Amend and Fender, 1976; Gould *et al.*, 1978)[88,89], the hyperosmotic infiltration (HI), direct immersion (DI) and spray methods were proved effective against ayu vibriosis (Aoki and Kitao, 1978; Nakajima and Chikahata, 1979; Kusuda *et al.*, 1980; Jo, 1981; Itami and Kusuda, 1978, 1980)[41,90,91,33,92-94]. The injection vaccination was tried just for the purpose of comparison with the foregoing methods (Aoki *et al.*, 1982, 1984)[95,35,96]. In addition to these studies which were mainly carried out at universities, exhaustive trials on oral and immersion vaccinations were made at several prefectural research stations from 1976 to 1981, and the results of these experiments were put together by Minami *et al.* (1983)[36]. The methods and results of some representative trials of these vaccination experiments in ayu vibriosis are summarized in three tables: Table 3 is for oral vaccination, Table 4 for immersion vaccination and Table 5 for spray and injecton vaccinations.

In oral vaccination experiments, the protection against experimental infection was brought about by administration of 0.1 g to 4 g (wet weight) of formalin-killed cells per kg (fish body weight)·day for 15 days. The minimal required doses were reported 0.1 g/kg·day (Kawai and Kusuda, 1983, 1985)[97,99] or 1-4 g/kg·day (Jo, 1981; Minami *et al.*, 1983)[33,36]. For the first time, the duration of oral vaccination was reported to be 4 weeks at longest (Kusuda *et al.*, 1978)[87], but later the efficacy was confirmed to last about 60 days (Nakajima and Chikahata, 1979; Kawai and Kusuda, 1985)[90,99].

On the mechanism of oral vaccination in ayu, Kawai *et al.* (1981)[65] reported that the agglutinin titer rose only in the body surface mucus of the immunized fish and that the detection rate of the pathogen on the body surface of the immunized fish in challenge tests was significantly lower than that of the non-immunized fish. They concluded that the efficacy of the oral vaccination was attributed mainly to the aggluninin secreted in the body surface mucus.

Table 3. Trials of oral vaccination against vibriosis in ayu

Author (year)	Immunogen	Dose	Protection (Challenge method)	Rise in antibody titer	Duration of efficacy
Kusuda *et al.* (1978)[87]	FKC, HC, SC, FKC+FIA	0.4, 4 g/kg·day ×1, 2, 4 w	Yes (WB)	No (serum)	4 w
Nakajima & Chikahata (1979)[90]	FKC	0.16 g×16 d	Yes (WB)	titer 8 (serum)	60 d
Jo (1981)[33]	FKC	1 g×15 d	Yes (WB)		
Minami *et al.* (1983)[36]	FKC	4 g×15 d	Yes (WB)		3−7 w
Kawai & Kusuda (1983)[97]	FKC LPS	0.1, 1 g 25 mg ×9 d	Yes (WB)		
Kawano *et al.* (1984)[98]	LC	0.4 g×15 d	Yes (WB)	No (serum)	50 d
Kawai & Kusuda (1985)[99]	FKC	0.1 g×8−15 d	Yes (NC, WB)		59 d

FKC: Formalin-killed cells, HC: Heated cells, SC: Sonicated cells, FIA: Freund's incomplete adjuvant, LPS: Lipopolysaccharide, LC: Lyophilized cells, WB: Water-born method, NC: Natural challenge, Duration: Days or weeks after the final administration of vaccine.

Table 4. Trials of immersion vaccination against vibriosis in ayu

Author (year)	HI or DI	Immunogen and Dose	Protection (Challenge method)	Rise in antibody titer	Duration of efficacy
Nakajima & Chikahata (1979)[90]	HI (7% NaCl)	FKB 10%×3 min	Yes (WB)	Yes (serum) (titer 32)	90 d
Kusuda *et al.* (1980)[91]	DI	FKB 100%×30 sec 0.05-0.5%×5 h	Yes (WB)		
Jo (1981)[33]	DI	FKC 0.1, 1 g/ℓ×10 min 0.1 g/ℓ×0.5-6 h	Yes (WB)		8 w
Aoki *et al.* (1982)[95]	HI (5.32% NaCl)	FKB 100%×2 min	Yes (IP)	No (serum)	48 d
Sumida (1983)[100]	DI	FKC 10^8 cells/mℓ×20 min	Yes (WB)		19 w
Minami *et al.* (1983)[36]	DI	FKC 1 g/ℓ×10 min 0.1 g/ℓ×0.5, 1 h 0.01 g/ℓ×24 h	Yes (WB)		2 m
Aoki *et al.* (1984)[35]	HI (5.32% NaCl)	FKB, FKC (10^7 cells/mℓ)×3 min ECP, LC (2 g/ℓ)×3 min	Yes (IP)	No (serum)	
Aoki *et al.* (1984)[96]	HI (5% NaCl)	FKC, SC, LC, LPS (10 μg/ℓ)×3 min	Yes (IP)	No (serum, skin mucus)	
Kawano *et al.* (1984)[98]	HI (5.32% NaCl)	LC (0.94, 9.4 g wet/ℓ)×3 min	Yes (WB)	No (serum)	113 d
Kawano *et al.* (1984)[101]	DI	LC (0.94, 9.4 g wet/ℓ)×4, 8 h	Yes (WB)		60 d

HI: Hyperosmotic infiltration, DI: Direct immersion, FKB: Formalin-killed broth, FKC: Formalin-killed cells, ECP: Extracellular products, LC: Lyophilized cells, SC: Sonicated cells, LPS: Lipopolysaccharides, WB: Water-born method, IP: Intraperitoneal injection

Table 5. Trials of spray and injection vaccinations against vibriosis in ayu

Author (year)	Immunogen and Dose	Protection (Challenge method)	Rise in antibody titer	Duration of efficacy
Spray				
Itami & Kusuda (1978)[92]	FKB (bentonite 0.15%, pH 3), 5-10 sec	Yes (WB)	Yes (serum) (titer 32)	
Itami & Kusuda (1980)[93]	FKB, FKB (bentonite 0.15%, pH 3), ECP, 5-7 sec	Yes (WB)	No (serum)	
Itami & Kusuda (1980)[94]	FKB, HC, SC, 5-7 sec	Yes (WB)	No (serum)	16 w
Injection (Intraperitoneal)				
Aoki *et al.* (1982)[95]	FKC, FKC+FCA 1.8×10^7 cells/fish	Yes (IP)	Yes (serum) (titer 4, 8)	
Aoki *et al.* (1984)[35]	FKC, FKC+FCA 1.8×10^7 cells/fish	Yes (IP)	Yes (serum 8, 32) No (skin mucus)	
Aoki *et al.* (1984)[96]	FKC+FCA 0.1 mℓ/fish	Yes (IP)	Yes (serum) (titer 64)	

FKB: Formalin-killed broth, ECP: Extracellular products, HC: Heated cells, FKC: Formalin-killed cells, FCA: Freund's complete adjuvant, WB: Water-born method, IP: Intraperitoneal injection.

The investigation into immersion vaccination in ayu were started according to the new vaccination system suggested by Amend and Fender (1976)[88], thus hyperosmotic infiltration (HI) method with 5.32, 5 or 7% NaCl solution in two steps or one step was employed first. However, HI method was found to give significant stress effects especially on young fish, and soon direct immersion (DI) method without hyperosmotic treatment was proved to be by no means inferior to HI method. In the early trials formalin-added broth culture was used as immunogen, and a toxic effect of the spent medium on smaller ayu was observed not only in HI method (Aoki *et al.*, 1982, 1984)[95,35] but also in DI method (Kusuda *et al.*, 1980)[91]. Then a suspension of the formalin-killed cells free from medium or lyophilized cells was chosen as the immunogen (Kawano *et al.*, 1984)[98,101].

Both in oral and immersion vaccinations, lipopolysaccharide (LPS) antigen extracted from *V. anguillarum* was confirmed to give enough protection, and it was concluded that the thermostable antigen (O-antigen) played the most important role as protective immunogen (Kawai and Kusuda, 1983; Aoki *et al.*, 1984)[97,96]. Recently, extracellular protease produced by *V. anguillarum* was demonstrated to play a protective immunogen by IP-injection vaccination in ayu and eels. However, when ayu was immunized with the enzyme by DI method, the efficacy of this protective immunogen was spoiled by the toxicity (Kanemori *et al.*, 1987)[102]. They concluded that the unwashed cells harvested from cellophane-overlaid agar plates, which contain O-antigen and an adequate amount of protease, gave better protection than the washed cells.

Various choices of concentration of vaccine and immersion period have been presented: for example, 1 g(wet weight) formalin-killed cells/ℓ ($=1$ mg/mℓ$=10^9$ cells/mℓ) for 10 min, 0.1 g/ℓ for a half to several hours, and 0.01 g/ℓ for 1-24 h (Jo, 1981; Minami *et al.*, 1983)[33,36]. Although the duration of immunity will depend on the doses, the efficacy provided by a single DI vaccination lasted about 2 months (Jo, 1981; Minami *et al.*, 1983; Kawano *et al.*, 1984)[33,36,101] or longer (Sumida, 1983)[100].

Aoki *et al.* (1984)[96] reported in a study on the mechanism of immersion vaccination in ayu that no agglutinin antibodies were detected in the serum or skin mucus of the vaccinated fish. Aoki *et al.* (1986)[103] suggested that the invading *V. anguillarum* might be eliminated by activated lymphocytes, neutrophils and macrophages through immersion vaccination. However, the whole mechanism of protection in ayu as well as that of the infection itself still remains to be solved.

Spray vaccination, a variation of immersion method, does not seem so practical for ayu, because the fish is very sensitive to handling stress.

Lastly, some minute points are discussed as below. In many experiments, strains belonging to J-O-1 serotype were used as immunogens and disease agent in challenge tests. Although polyvalent vaccines were tested in some studies on oral (Kusuda *et al.*, 1978; Kawano *et al.*, 1984)[87,98] and immersion vaccinations (Sumida, 1983; Kawano *et al.*, 1984)[100,101] and no antigenic competition was observed, cross protection between different serotypes has not been examined so far.

According to reported records, immersion vaccination succeeded in fingerlings of ayu as small as 0.176 g (120 day-old, Minami *et al.*, 1983)[36] and 0.15 g (Jo, 1981)[33], and oral vaccination resulted in good protection in smaller fish (0.063 g, 73 day-old, Yamamoto *et al.*, 1985)[104].

Since the serum antibody titer was not detected or raised in ayu vaccinated either by oral or immersion method, the evaluation of efficacy depends exclusively on challenge tests. In the early stages of the study, various challenge methods such as injections, direct contact and water-born methods were employed (Aoki *et al.*, 1982)[95]. But now, according to the detailed studies made by Jo (1981)[33], Kawano *et al.* (1983)[105] and others, the immersion challenge in 1% NaCl solution with 10^6 cells/mℓ of a virulent *V. anguillarum* strain for 3-5 min is adopted as the generalized method (Minami *et al.*, 1983)[36].

Most of vaccination experiments were carried out at water temperatures of 17-20°C, though some were performed at lower (10-17°C) or higher (20-26°C) temperatures (Minami *et al.*, 1983). Effects of water temperature on the efficacy of vaccination have not been examined due to the relatively narrow range of the temperatures employed.

CONCLUSION AND PERSPECTIVE

As far as the procurement of seedlings for pond culture depends on natural fingerlings, it seems impossible for ayu culture to be free from vibriosis, because seedling ayu, caught in either marine or freshwater environments, habor the pathogen at certain rates (Muroga *et al.*, 1984)[52]. Under these circumstances, business of ayu culture would not pay without the help of chemotherapy, or even with it. Thus, another control measures which will bring forth more stable and long-lived efficacy is wanted. Fortunately, immunological prophylaxis has been confirmed experimentally to be available for controlling vibriosis in ayu, though the mechanism of protection in vaccinated fish has yet to be elucidated.

When commercial vibrio vaccines are introduced into ayu farms, it may well be that the losses due to other infections such as *Streptococcus* and *Aeromonas* infections will increase, if culturists rely upon the vaccines too much. Another predictable problem is immunological suppressions produced by chemotherapeutics. For example, oxytetracycline was reported to give an immuno-suppressive effect in carp (Rijkers *et al.*, 1980; Van Muiswinkel *et al.*, 1985)[106,107].

So far as in ayu, immunological response induced by oral vaccination was not suppressed by oral administrations of oxolinic acid (Kawai and Kusuda, 1985)[99]. The effects of other chemotherapeutics on the immune response of ayu must be examined from now.

Recently, seed production of ayu has become prosperous in various prefectures. The seedlings produced are generally released into rivers to strengthen wild stocks and some are delivered to farms. Vibriosis also occurs in this seed production stage, and immunological prophylaxis is expected to work effectively in this stage. The youngest stage capable of immune response should be determined carefully as well as the development of lymphoid organs in ayu.

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(J): written in Japanese, (Je): in Japanese with English abstract, Others in English

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アユのビブリオ病（総説）

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アユ (*Plecoglossus altivelis*) のビブリオ病は *Vibrio anguillarum* を原因とする細菌性疾病であり，アユ養殖においては無論のこと，わが国の魚類養殖における最も被害の大きな疾病の一つに挙げられる。1967年に本病の発生が最初に報告されて以来，この20年間に本病およびその原因菌としての *V. anguillarum* に関し種々の研究がなされてきた。それらの成果を踏まえて，わが国では最初の魚類用ワクチンとして，本病に対する予防ワクチンが間もなく市販されるものと予測されるが，ここではこれまでに得られた本病に関する知見を整理してみた。

本病に罹ったアユより分離された *V. anguillarum* はアユのみでなくブリなどの海産魚やウナギにも病原性を示すが，それらの魚種の中ではやはりアユが最も高い感受性を示す。血清学的には３つのO血清型が存在し，特に淡水池で養成中のアユ病魚より分離される菌株のほとんどは J-O-1 型に属する。

本病は季節，環境，宿主の月令などにほとんど関係なく発生する。本病の感染源としては，淡水養殖過程に発生する場合は保菌魚が，海水中の種苗生産過程に発生する場合はワムシやブライン・シュリンプといった餌料生物が，それぞれ挙げられている。*V. anguillarum* のアユへの侵入門戸は皮膚であるとされているが，感染成立の機序については更に詳細なる検討が必要である。

本病の治療のためにサルファ剤や抗生物質の経口投与がなされてきたが，1973年より薬剤耐性株の出現により化学療法が困難な状況になり，1975年頃より予防ワクチンの開発のための研究が始められた。その結果，本菌のホルマリン死菌あるいは抽出 LPS を抗原とした経口免疫法，浸漬免疫法およびスプレー免疫法のいずれもが実験的には極めて有効であることが確かめられた。

今後は実際の養殖場における予防ワクチンの有効性について追跡するとともに，予防免疫におよぼす薬剤投与の影響，あるいは仔稚魚期への応用などについて更に検討される必要があるものと考えられる。