Fate and Location of *Vibrio anguillarum* in Tissues of artificially infected Ayu (*Plecoglossus altivelis*)

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Ayu (*Plecoglossus altivelis*) were infected with *Vibrio anguillarum* by a water-born method. At 6, 12, 18, 36, 38-45 (moribund stage) and 48 h (dead) after infection, fish were sampled to determine the fate and location of the bacterium in various tissues by viable cell count and the enzyme-labelled antibody technique (ELAT).

V. anguillarum was first detected in the skin at 12 h by bacterial isolation. It appeared in the muscle, spleen and liver at 24 h, but was not isolated from the gills or intestine until 36 h or 38-45 h. The same trend in the fate of the pathogen was confirmed by ELAT, and the cells were found in dermal layer of the skin from the early stage (12 h) of infection. Based on these observations, it was concluded that the first colonization site of *V. anguillarum* in ayu was the skin.

Vibriosis caused by *Vibrio anguillarum* is one of the most serious bacterial diseases of fish (EVELYN, 1984). In Japan, the disease has been reported to occur in ayu, *Plecoglossus altivelis* (MUROGA and EGUSA, 1967), rainbow trout, *Salmo gairdneri* (HOSHINA, 1956), Japanese eel, *Anguilla japonica* (JO and MUROGA, 1972), Yellowtail, *Seriola quinqueradiata* (JO *et al.*, 1979) and some other fresh water and marine fishes. It has been particularly devastating to populations of farmed ayu (JO, 1981), and various studies have been carried out on the infection in this anadromous fish. For example, studies on the histopathology of infected ayu (FUNAHASHI *et al.*, 1974) and mechanisms of protection in orally vaccinated fish (KAWAI *et al.*, 1981) have been conducted. Nevertheless, as in the case with salmonid fishes (NELSON *et al.*, 1985), little is known about the initial site of infection in the host.

In this study, the fate and location of the invading *V. anguillarum* in the tissues of ayu infected by a water-born method were investi-

gated by the ordinary bacterial plate count and the enzyme-labelled antibody technique. Consequently, it was revealed that the first colonization site of *V. anguillarum* in ayu was the skin.

Materials and Methods

Experimental animal and infection

Ayu which had been raised from juvenile stage in a pond in the campus of Hiroshima University were used in this study. Fish weighing 3 to 15 g were immersed for 5 min in 5 liters of distilled water containing 1% NaCl and 10^6 CFU/ml of *V. anguillarum* (PT-81049, serotype J-O-1). They were subsequently transferred to a rectangular plastic container of 30 liter capacity supplied with constant flow of de-chlorinated tap water. Temperature was maintained at about 20°C and fish were not fed during the course of experiments. After infection, fish were sacrificed at 6, 12, 18, 36, 38-45, and 48 h to estimate the viable cell number and the location of the bacterium in the tissues. Prior to operation, fish were gently swabbed on the body surface with alcohol-soaked cotton to lessen contamination with water bacteria.

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Table 1. Fate of *V. anguillarum* in the tissues of ayu infected by water-born method

Tissue	Log number of viable cells of <i>V. anguillarum</i> per 1 g of tissue					
	6	12	Time after infection (hours)			
			24	36	38-45 (Moribund)	48 (Dead)
Skin	—*	2.4	4.7	7.4	7.4	8.4
Muscle	—	—	2.5	5.6	6.8	7.5
Spleen	—	—	3.5	6.4	8.6	9.0
Liver	—	—	2.7	5.8	7.5	8.7
Gill	—	—	—	4.5	7.0	7.6
Intestine	—	—	—	—	7.4	6.9

* Not detected (less than 2).

Bacterial cell count

To determine the viable cell count in the skin, muscle, liver, spleen, gills and intestine, homogenates of each tissue sample were diluted with sterile saline and spread on nutrient agar. After 2 day-incubation at 25°C, *V. anguillarum* colonies were counted, and CFU per 1 g of each tissues was determined.

Enzyme-labelled antibody technique (ELAT)

Pieces of the tissues were fixed in 10% phosphate-buffered formalin at 4°C for 3-4 days. These were washed in distilled water, processed and embedded in paraffin of a melting point of 56°C. The embedded tissues were sectioned at 5 µm and stained using an enzyme-labelled antibody technique (WATANABE, 1981). A rabbit anti-serum prepared against heat-killed *V. anguillarum* (J-O-1) was used as the primary antibody. The secondary antibody labelled with peroxidase was purchased (Affinity purified antibody to rabbit IgG goat, Kirkegaard and Perry Lab., Inc. Maryland, USA).

Results

Fate of *V. anguillarum* determined by bacterial isolation

In order to examine the effects of size and degree of maturation of ayu on the susceptibility to *V. anguillarum*, the experiments on the fate of the pathogen were conducted 4 times at different stages of fish growth from June to September in 1985. Two fish were sacrificed at each sampling time in every experiment. The average body weight of material fish in 4 experiments were 6.6, 7.1, 7.0 and 13.4 g, respectively. The fish used in the third experiment in

Table 2. Location of *V. anguillarum* determined by an enzyme-labelled antibody technique in various tissues of ayu after water-born infection

Tissue	Time after infection (h)				
	6	12	24	36	38-45 (Moribund)
Skin	—	+	+	+	+
Muscle	—	—	+	+	+
Spleen	NE*	—	+	+	+
Liver	NE	—	+	+	+
Gill	NE	—	—	+	+
Intestine	NE	—	—	+	+

NE: Not examined, — absent, + present.

August showed developing gonads and those used in the last experiment in September were apparently matured. However, there were no differences in the results of *V. anguillarum* fate among the 4 experiments, and it became clear that size and degree of maturation did not influence the susceptibility of ayu to the pathogen. Thus, the results of the 4 experiments were put together in Table 1.

No signs of abnormalities in behaviour or in appearance were observed in infected ayu within 24 h after infection. The fish became lethargic and small petechial haemorrhages appeared on the body surface after 36 h or later. All fish were dead around 48 h after infection. The earliest appearance of *V. anguillarum* was observed 12 h after in the skin of half of the examined fish (4/8) at lower levels of viable cell counts with mean value of $10^{2.4}$ CFU/g, and the bacterial number at this site had been most prominent up to 36 h. In the muscle, spleen and liver the bacterial count became positive at 24 h,

and reached to the highest level in the spleen during moribund and death stages. Finally the organism appeared in the gills and intestine after 36 h or later and increased towards the last stage of infection.

Location of V. anguillarum determined by ELAT

In this experiment, 4 or 5 fish were submitted to the examination for each sampling time. The results are summarized in Table 2. No *V. anguillarum* cells were found either in the skin or muscle at 6 h. At 12 h after infection, scattered cells of the organism were observed in the dermal layer of the skin but no cells in any other tissues examined. After 24 h dense populations of the bacterium were seen sporadically along the dermal layer of the skin. the epidermis above the site where bacterial multiplication occurred was somewhat necrotized and sometimes disappeared. The organisms were abundant in the spleen, and several cells were observed near the blood vessels in the liver and between the bundle of the muscle fibers at this time. After 36 h the cells were found in all the tissues examined.

Discussion

The fate of pathogens in fish after artificial infection have been reported with various bacteria such as *Aeromonas hydrophila* (HATAI, 1972) *Edwardsiella tarda* (KUSUDA and ISHIHARA, 1981) *Pseudomonas anguilliseptica* (NAKAI *et al.*, 1985) and *V. anguillarum* (MUROGA, 1975) in eels, *A. salmonicida* in amago salmon, *Oncorhynchus rhodurus* (MORIKAWA *et al.*, 1981), *Streptococcus* sp. in yellowtail (KUSUDA and KIMURA, 1978) and *V. ordalii* in coho salmon, *O. kisutch* (SCHIEWE, 1983). These studies except for *V. ordalii* were carried out with fishes infected by injections, thus it does not seem so significant to compare the present result with those. Yet, the fact that the pathogens were concentrated in the spleen and kidney of the infected fishes is common to all the investigations. In the present study where the water-born method was employed, the bacterium was also highly concentrated in the spleen at least in the latter stage of infection. The spleen and kidney are the most active organs in the removal of foreign organ-

isms wherein macrophage and lymphoid cells are highly concentrated (BURAS *et al.*, 1985). Thus *V. anguillarum*, as in the case with other fish pathogens, is likely trapped and concentrated in melano-macrophage center in these organs.

The gills, skin and gastrointestinal tract have been implicated as the site of entry of *V. anguillarum* (EVELYN, 1984). Recently, NELSON *et al.* (1985) investigated the location of *V. anguillarum* in water-born infected rainbow trout using the fluorescent antibody technique. In their study, the pathogen could not be detected in the gills or gastrointestinal tract in the early stage of infection when the organism already appeared in the spleen and kidney. In our experiment, the pathogen also emerged in these organs only after the systemic infection was established.

An important finding of the present study is that *V. anguillarum* was first detected in the skin prior to any other tissues examined. The existence of the pathogen in this tissue was not examined by NELSON *et al.* (1985). Based on histopathological observations of ayu infected with *V. anguillarum*, FUNAHASHI *et al.* (1974) concluded that the first focal infection occurred in the skin. KAWAI *et al.* (1981) investigated the appearance of the pathogen in the skin and intestine in orally vaccinated and unvaccinated ayu after the water-born challenge. *V. anguillarum* was isolated from the skin of unvaccinated fish but not from that of vaccinated ones. These and other observations led them to conclude that the skin was the site of entry for *V. anguillarum* and that vaccination was efficacious because it prevented attachment of the pathogen to the skin. In the present study, though the exact invading stage itself was not demonstrated, it became clear that the first colonization site of the pathogen in ayu was the dermal layer of the skin. Although we can not ignore the possibility that the organisms penetrate into the host at the gills or intestine and promptly migrate to the dermal layer of the skin where the mobilization of defense factors such as leucocytes and serum components seems poor, the skin itself is most likely the entering site for the pathogen. As mentioned before, the body surface of the material fish were swabbed

in this study. In another our experiment where the fish were not wiped on their body surface, *V. anguillarum* was sometimes isolated from the skin at 6 h and even at 1 h after infection. The pathogen could not be observed in those samples by ELAT, and it may be due to the defecation of epidermal or mucous layer of the skin during the process of ELAT.

Based on the present result and some other past findings the pathogenesis of vibriosis in ayu could be described as follows: When virulent *V. anguillarum* cells come across the host fish, the organisms first attach themselves on the surface of the skin. Although most of them will be killed or inactivated by non-specific antibacterial activities of skin mucus (ITAMI *et al.*, 1986), some cells can penetrate into the dermal layer and proliferate there if they successfully escape from surveillance system of the host defense mechanism. Upon proliferation, the excess organisms are conveyed to various organs through blood streams, and induce the systemic infection. Toxic substances like protease (INAMURA *et al.*, 1984, 1985) continuously produced by the proliferated cells will drive the host to the death. Considering that only 10 cells of the pathogen introduced by intramuscular injection can kill ayu (Jo and MUROGA, 1977), whether the organisms can penetrate through the skin surface or not seems to be the most crucial point in the development of the infection.

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