The Tissue Distribution of Atypical *Aeromonas salmonicida* in Artificially Infected Japanese Eels, *Anguilla japonica*

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The multiplication and distribution of an atypical *Aeromonas salmonicida* strain, which was isolated as a causative agent of "head ulcer disease" of cultured eels, in artificially infected Japanese eels (*Anguilla japonica*) were studied by bacteriological culture technique. When the eels were injected intramuscularly with a lethal dose of the atypical *A. salmonicida* strain at 20°C, the pathogen was recovered at high concentrations (10^7–10^9 CFU/g) from the muscle of the injected site throughout the course of infection. But in the spleen and kidney very small numbers of the pathogen were detected even at the moribund stage, and the blood and brain were almost aseptic. A similar result was obtained in the eels which were artificially injured on the skin and infected with the pathogen. These data indicate that the disease does not attain to distinct septicemic condition but the local proliferation of the pathogen will be the death of the host fish, and this pathological feature is in a striking contrast to other bacterial infections of eels. It was also found that the bacterial growth was significantly inhibited both *in vitro* and *in vivo* at 30°C, which uggests that temperature manipulation of pond water will be an effective measure to control the disease.

Furunculosis caused by *Aeromonas salmonicida* is one of the most widely distributed bacterial diseases of cultured fishes, especially salmonids, and the disease usually falls into systemic condition (McCarthy and Roberts, 1980). Recently, infections caused by the so-called atypical type of *A. salmonicida* have been reported in salmonid and non-salmonid species (McCarthy, 1977; Paterson *et al.*, 1980). Clinical features of these infections due to atypical *A. salmonicida* are generally characterized by the ulceration of the skin, and bacteremia develops only in the terminal stage, if any (Elliott and Shotts, 1980; Gayer *et al.*, 1980).

In Japan, an eel disease, namely "head ulcer disease", characterized by the conspicuous ulcer on the head and/or body surface has been prevailing in eel farms during the period of cold water temperature, and it was confirmed to be a bacterial infection. At first, *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Vibrio* sp. were con-

sidered to be the causative organisms (Hidaka *et al.*, 1983). Later, a fastidious organism was isolated from diseased eels as the causative agent, and it was classified as atypical *A. salmonicida* (Ohtsuka *et al.*, 1984; Iida *et al.*, 1984; Kitao *et al.*, 1984).

As noted in our previous paper (Ohtsuka *et al.*, 1984), the agent was rarely isolated from the blood and internal organs of naturally infected eels even at the progressed stage of the infection, though it was easily isolated from the skin or muscle of ulcerative lesions. This indicates that the fish became fatal without falling into systemic infection. In order to clarify further the fate of the pathogen, we examined here the tissue distribution of atypical *A. salmonicida* in artificially infected Japanese eels (*Anguilla japonica*) by bacteriological culture technique.

Materials and Methods

Experimental fish

Healthy Japanese eels (mean body weight: 80 g) were purchased from private fish farms with no history of "head ulcer disease". During the course of each experiment, the fish received no food.

Bacterial strain and culture medium

A strain ET-83205 of atypical *A. salmonicida* isolated from a diseased Japanese eel in 1983 was used in this study. After several fish passages, the strain attained high virulence to eels, the median lethal dose (LD_{50}) by intramuscular injection being less than 10^3 colony forming unit (CFU) per 100 g of fish.

An improvement of the medium for viable cell count of ET-83205 strain was made because of its fastidious growth on commercial agar media. Ishiguro *et al.* (1986) revealed that heme enhanced the plating efficiencies of atypical starins of *A. salmonicida* which were isolated from goldfish, carp, and Atlantic salmon. To confirm a similar effect of heme on the present eel-strain, hemin (Sigma) was added to tryptosoya agar (TSA, Nissui), brain heart infusion agar (BHIA, Eiken), heart infusion agar (HIA, Eiken), and nutrient agar (NA, Eiken). A preculture of ET-83205 strain was serially diluted with saline containing 0.01 % of tween 80 (saline-tween) and each 0.1 ml of the dilutions was spread on each medium with or without hemin (10 µg/ml). The viable cell count was made after 3 day incubation at 25°C.

Effect of temperature on the bacteial growth

The strain was inoculated in hemin (10 µg/ml)-containing trypticase soy broth (BBL) at a concentration of $10^{6.1}$ CFU/ml and shake-cultured at various temperatures ranging from 15°C to 37°C. The growth was monitored photometrically (Optical Density) up to 150 hours post-inoculation.

Infection methods

Fish were anesthetized with 1.5% urethan and infected with a 3 day culture of ET-83205 strain by either of the following two methods. In one experiment, fish were injected with the bacteria into the trunk muscle at a dose of $10^{8.8}$ CFU/100 g,

and then kept in freshwater tanks at 20°C or 30°C for 10 days. In another experiment, fish were injured on the head skin by scissors, and then 20 µl of the bacterial suspension ($10^{9.0}$ CFU/ml) was applied to the injured site. After 2 min of exposure, fish were kept in tanks at 20°C for 30 days.

Viable cell count in the infected eels

After inoculation of the pathogen, four eels from each experimental group were sacrificed at adequate intervals for viable cell count of the tissues. The blood taken from the bulbus arteriosus by heparinized syringe and the homogenates of tissues (spleen, kidney, brain, and muscle with skin of infected site) were serially diluted by 10-fold with saline-tween, and each 0.1 ml of the dilutions was spread on TSA containing hemin (10 µg/ml). The number of viable cells per 1 g of tissue or 1 ml of blood was determined after 3 day incubation at 25°C.

Results

Effect of hemin on the viable cell count

Table 1 shows that the addition of hemin gave higher numbers in the viable cell count on every agar medium and hemin-containing TSA seemed to be the most preferable medium for the present purpose. The addition of hemin also accelerated the growth of colony and it led to the reduction in time for viable cell count. It was also found that the addition of tween 80 in diluent (saline) was indispensable to obtain more accurate result, possibly by inhibiting the autoaggregation of the organism.

Table 1. Effect of hemin on the viable cell count of atypical *Aeromonas salmonicida*

Agar medium	Hemin (10 µg/ml)	Log CFU per mg of inoculum*
TSA	+	8.8
	−	7.1
HIA	+	8.6
	−	7.3
BHIA	+	8.4
	−	7.2
NA	+	8.4
	−	7.0

* Assayed after incubation at 25°C for 3 days.
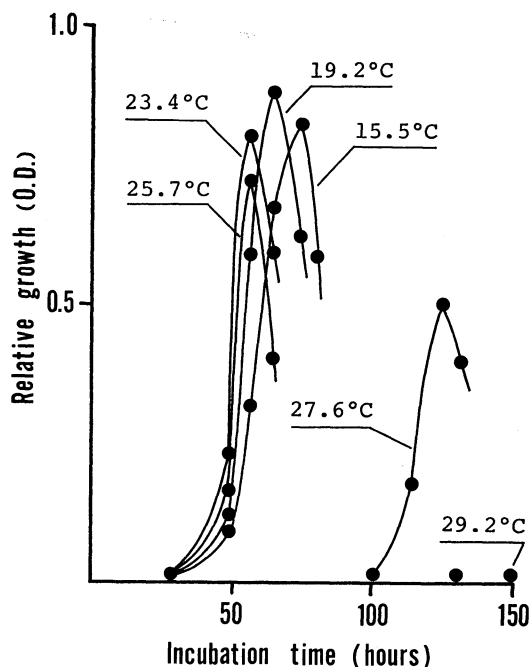
Fig. 1. Effect of temperature on the growth of atypical *A. salmonicida*.

Fig. 2. Changes in number of the viable cells in Japanese eels injcted intramuscularly ($10^{8.8}$ CFU/100 g) with atypical *A. salmonicida* at 20°C. Muscle of injected site (●), Blood (○), Spleen (△), Kidney (▲), Brain (■). Bars represent range ($n=4$).

Effect of temperature on the growth

The relative growth of the bacteria at various temperatures is presented in Fig. 1. Although the fastest growth was seen at 23.4°C and 25.7°C, the final cell concentration was little different at temperatures between 15.5°C and 25.7°C. However, the growth at 27.6°C was inhibited significantly in respect to the prolonged lag time and lower final cell concentration, and no growth was seen at 29.2°C during 150 h incubation. A rapid decrease of optical density after the maximum growth at each temperature was observed owing to the formation of cell clumps.

Fate of the organism in the infected eels

Figure 2 shows the changes in log number of the viable cells in the tissues of the eels injected intramuscularly at 20°C (19.4–20.3°C). The fish died 4–6 days (mean 5.1 days) after injection, showing heavy hemorrhage and abscess in the muscle of the injected site and congestion in the gills. A slight swelling of the spleen and kidney was also observed in the dead fish. The injected bacteria were detected from the spleen and kidney
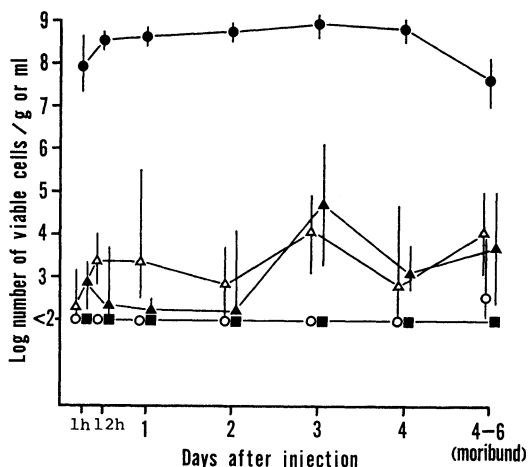
of some fish during the period of infection experiment, but the mean number of the cells remained at considerably low levels ($10^{2.1}$–$10^{4.8}$ CFU/g). It should be noticed that the blood and brain were almost aseptic (less than the limit of detection, 10^2 CFU/ml or g) except for the blood at the moribund stage ($10^{2.3}$ CFU/ml). The existence of the pathogen or phagocytosis by leukocytes was not observed in stained samples of blood smear at any stages. In contrast, high numbers of the bacterial cells were demonstrated constantly in the injected muscle layers ($10^{7.6}$–$10^{8.9}$ CFU/g).

The fate of the organism in the eels injected intramuscularly at 30°C (29.9–30.4°C) is shown in Fig. 3. Very low numbers of the cells were detected from the spleen and kidney of some eels at day-1 and they became undetectable at day-3. The blood and brain were always aseptic. The bacterial number in the injected muscle also decreased from $10^{8.0}$ CFU/g at day-1 to $10^{3.2}$ CFU/g at day-5. Fish were all alive and any remarkable changes were not seen at day-10.

In a group of eels, which were applied the pathogen to the injured site of head skin at 20°C, fish died 11–20 days (mean 14.0 days) after inoculation and the mortality reached 67%. Dead fish showed serious hemorrhage and abscess
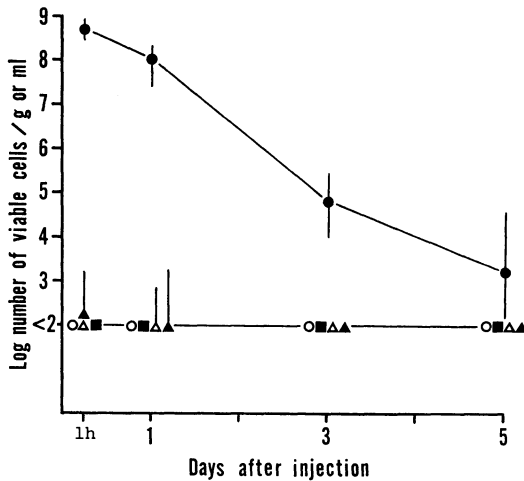
Fig. 3. Changes in number of the viable cells in Japanese eels injected intramuscularly ($10^{8.8}$ CFU/100 g) with atypical *A. salmonicida* at 30°C. Muscle of injected site (●), Blood (○), Spleen (△), Kidney (▲), Brain (■). Bars represent range ($n=4$).
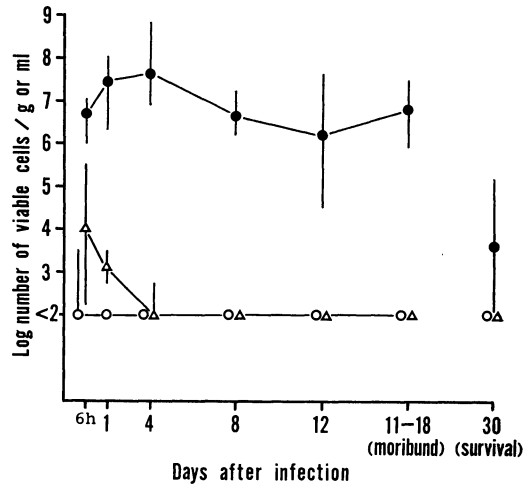
Fig. 4. Changes in number of the viable cells in Japanese eels infected with atypical *A. salmonicida* at 20°C. Fish were injured on the head skin by scissors and 20 μl of the cell suspension ($10^{9.0}$ CFU/m*l*) was applied to the injured site. Muscle of infected site (●), Blood (○), Spleen (△). Bars represent range ($n=4$).

at the skin and muscle of the inoculated site, heavy conjestion in the gills, a slight swelling of the spleen and kidney. The pathogen was not recovered from the spleen and blood at day-8 and thereafter, but high numbers ($10^{6.2}$–$10^{7.6}$ CFU/g) of the cells were detected from the muscle of the infected site during the course of infection (Fig. 4). From the affected site at the progressed stage, *A. hydrophila* was isolated with high frequency and high numbers (10^6–10^7 CFU/g) in addition to the inoculated atypical *A. salmonicida*. At day-30, when the experiment was terminated, the cell number of both *A. salmonicida* and *A. hydrophila* in the survivors decreased to a level of 10^3 CFU/g.

Discussion

Recently, a new subspecies name *nova* was proposed to atypical *Aeromonas salmonicida* strains isolated from carp and goldfish (Belland and Trust, 1988). Strains from eels, however, have not been compared genetically with the representative strains of *A. salmonicida* subsp. *nova*. Therefore, the strain used in this study was called just atypical *A. salmonicida*.

The present results support a previous finding (Ohtsuka et al., 1984) that "head ulcer disease" of eels caused by atypical *A. salmonicida* does not fall into systemic infection. A significant bacterial proliferation was seen only in the muscle of the infected site and the viable cell count of the pathogen in the spleen and kidney remained at low levels throughout the course of infection. The blood and brain were almost aseptic. Therefore, the failure of bacterial isolation from the internal organs of naturally infected eels is due to the low number of the pathogen in these tissues.

Experimental infections of Japanese eels with *Vibrio anguillarum* (Muroga, 1975), *Edwardsiella tarda* (Kusuda and Ishihara, 1981), and *Pseudomonas anguilliseptica* (Nakai et al., 1985) demonstrated that these were septicemic from the early stage and developed into systemic condition as summarized in Table 2. The feature of the disease process reproduced by the present study with atypical *A. salmonicida* is local infection, which is quite different from those by other eel-pathogenic bacteria.

Atypical *A. salmonicida* infections of goldfish

Table 2. The fates of atypical *A. salmonicida* and some other eel-pathogenic bacteria in the intramuscularly injected Japanese eels

Bacterium	Injected dose	Water temp. (°C)	Time after injection	Log CFU/m*l* or g			Reference
				Blood	Spleen	Kidney	
atypical *Aeromonas*	$10^{8.8}$	20	1 h	<2.0	2.3	2.9	Present study
salmonicida	cells		12 h	<2.0	3.4	2.2	
			4 d	<2.0	2.8	3.1	
Vibrio anguillarum	1 mg	17.5	6 h	5.9	6.8	5.5	MUROGA (1975)
			36 h	6.5	6.9	6.2	
Edwardsiella tarda	$10^{7.9}$	26	1 h	5.0	7.0	7.0	KUSUDA & ISHIHARA
	cells		12 h	5.9	7.6	7.0	(1981)
			48 h	6.6	8.8	8.0	
Pseudomonas	$10^{9.0}$	20	1 h	6.4	6.8	6.2	NAKAI *et al.* (1985)
anguilliseptica	cells		12 h	4.5	5.3	3.7	
			7 d	9.2	10.1	9.2	

(ELLIOTT and SHOTTS, 1980) and carp (GAYER *et al.*, 1980; CSABA *et al.*, 1981) were also described as ulcerative local infections, though the details of the fate of the pathogen were not shown in these fishes. Thus, this pathological feature seems to be common to atypical strains of *A. salmonicida*. However, the factors contributing to the death of these fishes infected with atypical *A. salmonicida* remain unknown.

Another finding in our previous study on the disease is a fact that the atypical *A. salmonicida* could not kill Japanese eels when the fish were injected intramuscularly and kept at 30°C. The present study confirmed that the *in vitro* growth of the pathogen was inhibited remarkably at higher temperatures than 27°C and the pathogen disappeared rapidly from the tissues of infected eels kept at 30°C. As already anticipated in our previous paper, to keep the pond water at 27°C or higher was established as an effective control measure for this disease.

In one experiment of the present study, the disease was reproduced by exposure of the injured site of the skin to bacterial suspension. This result suggests that an injury on the body surface is of importance as a portal of the pathogen.

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