魚病研究 Fish Pathology 19 (4) 253-258, 1985. 3

The fate of *Pseudomonas anguilliseptica* in Artificially Infected Eels *Anguilla japonica*[*1]

Toshihiro NAKAI, Yoshifumi KANEMORI, Kazuhiro NAKAJIMA, and Kiyokuni MUROGA

Faculty of Applied Biological Science, Hiroshima University, Fukuyama, 720 Japan

(Received September 22, 1984)

The multiplication and distribution of *Pseudomonas anguilliseptica* in artificially infected Japanese eels were studied by viable cell count.

When the eels were injected intramuscularly with the lethal doses (10^7, 10^9/100 g fish weight) of a K antigen-possessing virulent strain at 12°C or 20°C, the viable cell numbers in blood and tissues decreased from 1 h to 12 h post-inoculation. This phase was followed by a static phase, in which the organism persisted at lower concentration or grew slowly, and the duration of this phase depended on the inoculated dosage and ambient water temperature. At the final phase, the organism appeared in rapidly increasing number and rose to high levels (10^{8-10} viable cells/g or m*l*), which persisted until the death of fish.

On the contrary, when inoculated with sublethal dose (10^5 CFU) of the virulent strain, with lethal dose of virulent strain at a higher water temperature (28°C), or with a dose of a K antigen-lacking avirulent strain, the organism disappeared completely from the tissues except the muscle of the inoculated site within 1–3 days.

The final level of viable cell count in the moribund eels was higher than those of *Aeromonas hydrophila* or *Vibrio anguillarum*, and this difference was thought to reflect toxin production.

Pseudomonas anguilliseptica is known to cause red spot disease in eels, *Anguilla japonica* and *A. anguilla*, not only in Japan (WAKABAYASHI and EGUSA, 1972; Jo *et al.*, 1975) but also in Taiwan (KUO and KOU, 1978) and Scotland (NAKAI and MUROGA, 1982; STEWART *et al.*, 1983). In the previous serological studies (NAKAI *et al.*, 1981, 1982), we confirmed that the Japanese strains of *P. anguilliseptica* shared a common heat-stable antigen (O antigen) but could be divided into two serotypes based on the presence of heat-labile K antigen. It was also found that the strains possessing K antigen (K^+ type) were solely pathogenic to eels, though both of the types were originally isolated from diseased eels.

The present paper describes the multiplication and distribution of *P. anguilliseptica* in Japanese eels injected with various doses of K^+ strain and K^- strain under different water temperatures.

[*1] Studies on Red Spot Disease of Pond-cultured Eels—X.

Materials and Methods

Healthy Japanese eels (mean body weight: 110 g) obtained from private culture-ponds with no history of red spot disease were acclimated to each experimental water temperature for a few days. During the course of every experiment, the fish received no food.

Strain ET-7601 of K^+ type and strain ET-2 of K^- type of *P. anguilliseptica* were used as in the previous studies (NAKAI *et al.*, 1981, 1982). The cells of each strain cultured on nutrient agar (Eiken) at 20°C for 2 days were suspended in saline at various concentrations.

The eels were injected with the cell suspension intramuscularly at the base of dorsal fin (IM-injection) or intravenously into the bulbus arteriosus (IV-injection). The inoculating doses and water temperatures of experimental aquaria were designed as follows: for strain ET-7601, a) IM-injection with doses of 1×10^9, 1×10^7, and 1×10^5 CFU (colony forming unit) per 100 g fish weight at 20°C (18.5–21.0°C), b) IM-injection with

1×10^9 CFU at 12°C (10.1–14.0°C) and 28°C (27.0–29.0°C), c) IV-injection with 1×10^9 CFU at 20°C (19.1–20.1°C); and for strain ET-2, IM-injection with 1.5×10^9 CFU at 20°C (20.5–21.5°C).

After inoculation, two to four eels from each experimental group were sacrificed at adequate intervals for viable cell count in blood, liver, spleen, posterior kidney, and two sites of muscle including the skin (IS: muscle of injected site and RS: muscle remote form IS by about 10 cm). The blood samples drawn from the bulbus arteriosus and homogenates of each tissue sample were diluted in serial 10-fold increments in saline and spread on nutrient agar containing 0.1% $MgCl_2\cdot6H_2O$.

The agglutinin titers in eel plasma were also measured by microtiter technique using formalinized cells of each strain.

Results

Fig. 1A shows the changes in viable cell number in the eels IM-injected with 1×10^9 CFU of strain ET-7601 at 20°C. The bacteria appeared in all tissues 1 h after inoculation, but decreased slightly in number at 12 h post-inoculation. After that, the number of cells increased continuously at high rate in all the tissues except IS-muscle. At day 7, the numbers reached $10^{10.1}$/g in liver and spleen, $10^{9.2}$/g or m*l* in kidney and blood, and $10^{8.7}$/g in RS-muscle of moribund fish. In IS-muscle, the number of cells decreased gradually from $10^{8.4}$/g at 1 h to $10^{6.7}$/g at 5 d or 6 d post-inoculation, followed by a rapid increase in moribund state ($10^{8.8}$ at day 7). Hemorrhage developed in the skin and muscle around the injected site at 3 d post-inoculation, and moribund fish showed petechial hemorrhage on the whole body surface and congestion in the liver. Similar changes in the cell number were observed in the eels IV-inoculated with the same dose of the virulent strain under the same water temperature (Fig. 2).

In IM-inoculation with 1×10^7 CFU of strain ET-7601 (Fig. 1B), the bacterial cells in blood and tissues, after a temporary decrease at 12 h post-inoculation, persisted at low concentrations or increased at very slow rate until day 5. During this static period, the bacteria were not detectable ($<10^2$/g) in the splanchnic tissues of some individuals. This period was followed by a continuous increase in cell number. At day 10, the numbers reached $10^{7.8-9.7}$/g or m*l* in each tissues. The first death of fish occurred at day 8 and the others died by day 14.

In contrast, in the eels IM-inoculated with

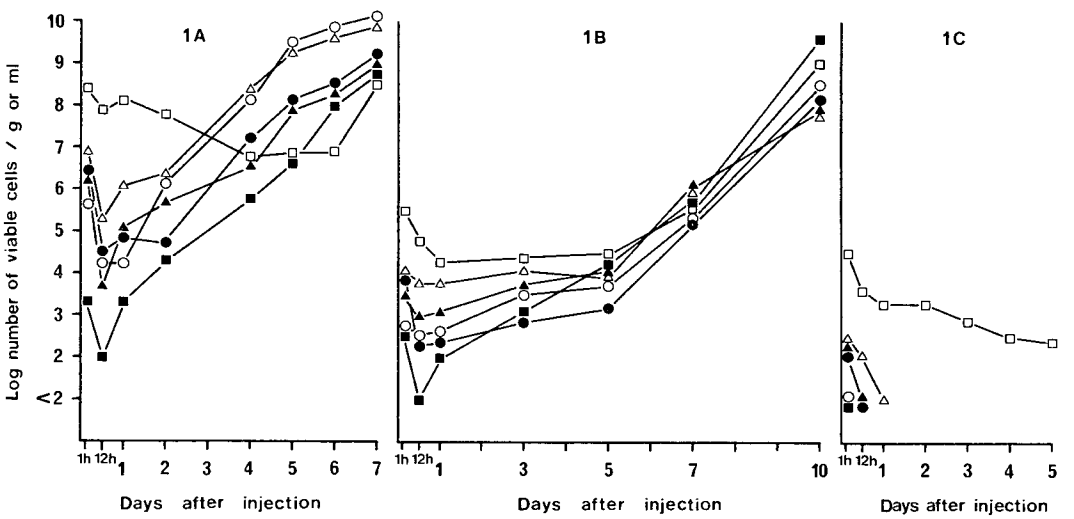

Fig. 1. Changes in number of viable cells in Japanese eels injected intramuscularly with 1×10^9 CFU (1A), 1×10^7 CFU (1B), and 1×10^5 CFU (1C) of *Pseudomonas anguilliseptica* strain ET-7601 at 20°C. Blood (●), Liver (○), Kidney (▲), Spleen (△), IS-Muscle (□), RS-Muscle (■).

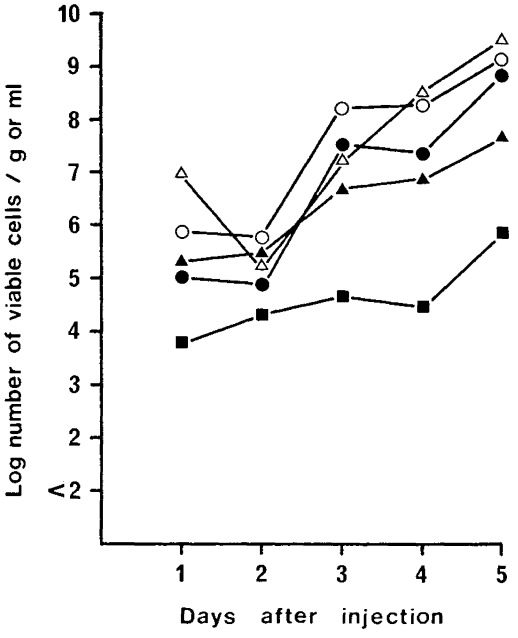

Fig. 2. Changes in number of viable cells in eels injected intravenously with 1×10^9 CFU of *P. anguilliseptica* strain ET-7601 at 20°C. Blood (●), Liver (○), Kidney (▲), Spleen (△), Muscle (■).

1×10^5 CFU, a swift clearance of the organism from every tissue except IS-muscle was brought about within 1 d, with no re-emergence thereafter (Fig. 1C). No death of fish occurred.

The changes in cell number in the eels IM-inoculated with 1×10^9 CFU of strain ET-7601 at 12°C and 28°C are shown in Fig. 3A and 3B, respectively. When fish were kept at 12°C, the temporary decrease was observed at/until day 3, followed by a slow rise in number, and the cell levels reached $10^{9.7}$/g in spleen and liver, $10^{9.0}$/ml or g in blood and IS-muscle, and $10^{8.5}$/g in kidney and RS-muscle 12 d after inoculation. Fish died from 13 d to 15 d post-inoculation. In contrast, the cell numbers of eels kept at 28°C decreased so swiftly that the bacteria disappeared completely from blood and tissues by day 2 and even from the IS-muscle by day 5. No fish died throughout the observation period of 10 days and any apparent symptoms were not noticed in the survivors.

In any of the plasma samples obtained from the eels inoculated with strain ET-7601 at 12°C and 20°C, no significant increase in agglutinin levels were detected against ET-7601 antigen (<4) or

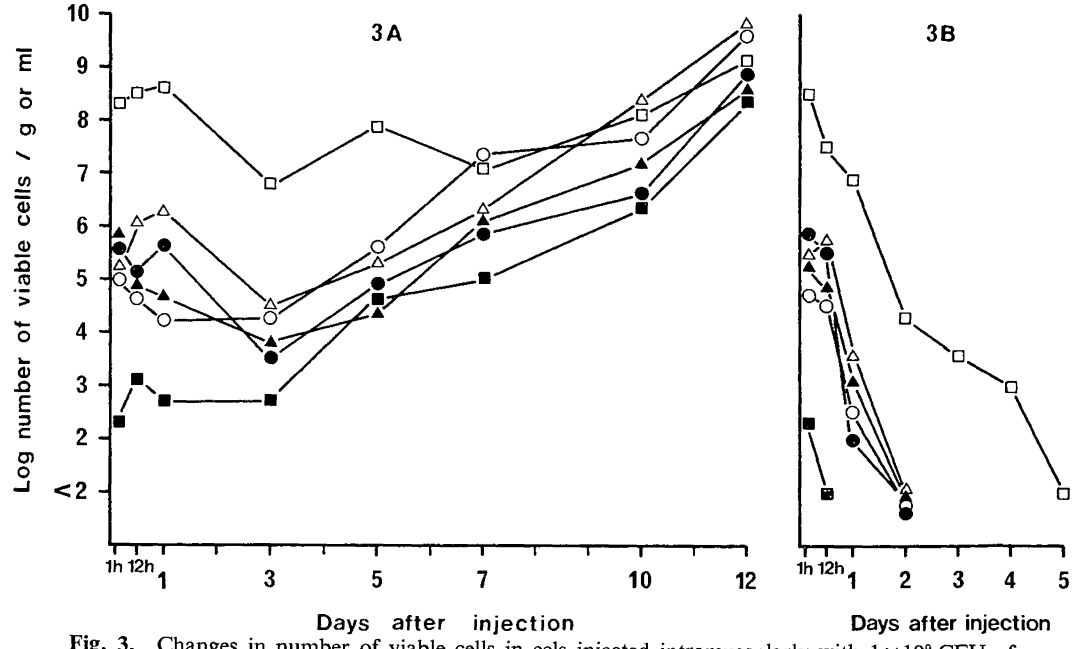

Fig. 3. Changes in number of viable cells in eels injected intramuscularly with 1×10^9 CFU of *P. anguilliseptica* strain ET-7601 at 12°C (3A) and 28°C (3B). Blood (●), Liver (○), Kidney (▲), Spleen (△), IS-Muscle (□), RS-Muscle (■).

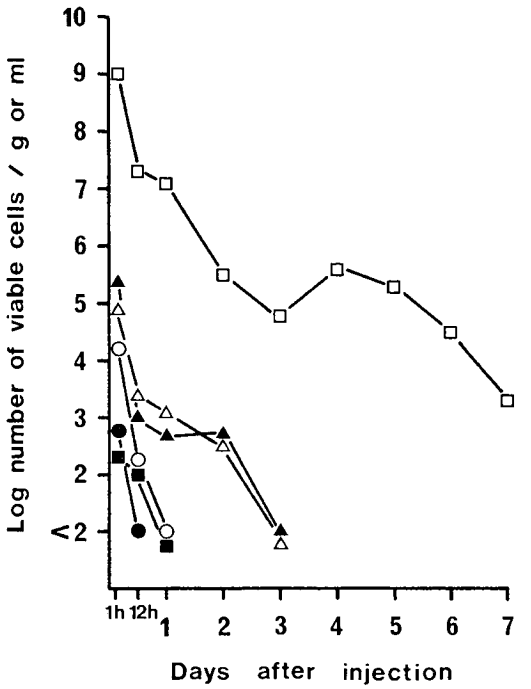

Fig. 4. Changes in number of viable cells in eels injected intramuscularly with 1.5×10^9 CFU of *P. anguilliseptica* strain ET-2 at 20°C. Blood (●), Liver (○), Kidney (▲), Spleen (△), IS-Muscle (□), RS-Muscle (■).

ET-2 antigen (<4–8). In the eels kept at 28°C, the agglutinin antibody was detected in the higher level than in control at 5 d post-inoculation and the level reached 32 against ET-7601 antigen or 256 against ET-2 antigen at day 7.

On the other hand, when the eels were inoculated intramuscularly with 1.5×10^9 CFU of strain ET-2 under 20°C, the viable cells disappeared completely from blood at 12 h post-inoculation, from liver and RS-muscle at 1 d, and from spleen and kidney at 3 d (Fig. 4). During the observation period of 10 days, no fish died and no disease symptoms were noticed. Agglutinins were not produced within the observation period of 7 days.

Discussion

When large number of living bacteria are injected into the veins of an experimental animal, subsequent clearance or bacteremia process monitored by viable cell count in the blood was reported to be divided into three phases (ROGERS, 1960). In the first phase, 90 to 99% of the circulating bacteria disappear, and this initial removal process appears essentially independent of the nature of the bacteria, the animal, or the subsequent outcome of infection. This phase is followed by a phase in which microorganisms persist in the circulation at lower concentrations, or by their more gradual removal at slower rates. In the third phase, non-pathogenic organisms or avirulent strains disappear completely from the blood stream. However, the bacteria producing fatal infection reappear in increasing numbers, and bacteremia rises to high levels which persist until the death of the animal.

The above is the generalized pattern of clearance of bacteria from the blood stream in mammals. The same pattern was basically observed not only in the blood but also in tissues of the eels injected intramuscularly or intravenously with *P. anguilliseptica*. That is, when inoculated with 10^7–10^9 CFU of the virulent strain at 12°C or 20°C, bacteremia developed finally in the eels. On the other hand, when inoculated with 10^5 CFU of the virulent strain or 10^9 CFU of the avirulent strain at 20°C, the bacteria were removed completely from the blood and tissues.

As presented in previous papers (MUROGA *et al.*, 1973, 1975), the outcome of the *P. anguilliseptica* infection, either naturally or experimentally, depends on ambient water temperature, resulting in no or quite low mortalities of eels at 27°C or above. The results of the present study that the injected organism was rapidly cleared from the eels kept at 28°C corresponded to this phenomenon. Although this phenomenon had been attributed mainly to the inactiveness of the organism at such higher temperatures (MUROGA *et al.*, 1977), it was also found in vitro experiments that eel normal serum lysed the cells of *P. anguillisptica* more effectively at 28°C than at 20°C (Data will be presented in another paper). Therefore, it seems possible that the nature of the organism and a host defense mechanism are both involved in this phenomenon.

In mammals (ROGERS, 1960), it was revealed that the inoculated bacteria were recovered at the highest numbers from the liver and spleen,

and these organs played an important role in clearance of the organisms from the body. Also in fishes, some reports (HATAI, 1972; MUROGA, 1975; KUSUDA and KIMURA, 1978; KIMURA and KUSUDA, 1979; KUSUDA and ISHIHARA, 1981) descrived that inoculated pathogens were highly concentrated in the kidney and/or spleen. In the present study, however, the kidney and spleen were not always the organs where the bacteria were concentrated at the highest level. Especially in the moribund eels, the viable cells of *P. anguilliseptica* were detected at almost the same high concentrations (10^{8-10}/g or m*l*) in any of the tissues examined. Similar vigorous multiplication in eels was reported in *Edwardsiella tarda* infection (KUSUDA and ISHIHARA, 1981), but not in *Aeromonas hydrophila* (HATAI, 1972) or *Vibrio anguillarum* (MUROGA, 1975) infections, though all were fatal to eels. The viable cell counts in moribund eels reported were 10^{7-9}/g or m*l* for *E. tarda* and 10^{8-7}/g or m*l* for *A. hydrophila* and *V. anguillarum*. One possible explanation for the difference in the degree of bacteremia by different eel pathogens may be the contribution of exotoxins and/or endotoxins lethal to eels. The latter two species were confirmed to produce toxins (SHIMIZU, 1969; INAMURA *et al.*, 1984), while *P. anguilliseptica* and *E. tarda* did not produce lethal toxins to eels in our experiments (data not shown).

This work was partly supported by research grants from the Ministry of Education.

References

HATAI, K. (1972): Studies on the fate of intravascularly injected bacteria in fish—I. Changes in number of cells of *Aeromonas liquefaciens* in the blood, liver, and kidney of eel. *Fish Pathol.*, **7**, 26–33.

INAMURA, H., K. MUROGA, and T. NAKAI (1984): Toxicity of extracellular products of *Vibrio anguillarum*. *Fish Pathol.*, **19**, 89–96.

JO, Y., K. MUROGA, and K. ŌNISHI (1975): Studies on red spot disease of pond cultured eels—III. A case of the disease in the European eels (*Anguilla anguilla*) cultured in Tokushima prefecture. *Fish Pathol.*, **9**, 115–118.

KIMURA, H. and R. KUSUDA (1979): Studies on the pathogenesis of streptococcal infection in cultured yellowtails *Seriola* spp. Effect of the cell free culture on experimental streptococcal infection. *J. Fish Diseases*, **2**, 501–510.

KUO, S. C. and G. H. KOU (1978): *Pseudomonas anguilliseptica* isolated from red spot disease of pond-cultured eel, *Anguilla japonica*. *Report of the Institute of Fishery Biology of Ministry of Economic Affairs and National Taiwan University*, **3**, 19–23.

KUSUDA, R. and S. ISHIHARA (1981): The fate of *Edwardsiella tarda* bacteria after intramuscular injection of eels, *Anguilla japonica*. *Bull. Japan. Soc. Sci. Fish.*, **47**, 475–479.

KUSUDA, R. and H. KIMURA (1978): Studies on the pathogenesis of streptococcal infection in cultured yellowtails *Seriola* spp. The fate of *Streptococcus* sp. bacteria after inoculation. *J. Fish Diseases*, **1**, 109–114.

MUROGA, K. (1975): Studies on *Vibrio anguillarum* and *V. anguillarum* infection. *J. Fac. Fish. Anim. Husb. Hiroshima Univ.*, **14**, 101–215.

MUROGA, K., Y. JO, and T. SAWADA (1975): Studies on red spot disease of pond-cultured eels—II. Pathogenicity of the causative bacterium, *Pseudomonas anguilliseptica*. *Fish Pathol.*, **9**, 107–114.

MUROGA, K., Y. JO, and M. YANO (1973): Studies on red spot disease of pond-cultured eels—I. The occurrence of the disease in eel culture ponds in Tokushima prefecture in 1972. *Fish Pathol.*, **8**, 1–9.

MUROGA, K., T. NAKAI, and T. SAWADA (1977): Studies on red spot disease of pond-cultured eels—IV. Physiological characteristics of the causative bacterium, *Pseudomonas anguilliseptica*. *Fish Pathol.*, **12**, 33–38.

NAKAI, T. and K. MUROGA (1982): *Pseudomonas anguilliseptica* isolated from European eels (*Anguilla anguilla*) in Scotland. *Fish Pathol.*, **17**, 147–150.

NAKAI, T., K. MUROGA, and H. WAKABAYASHI (1981): Serological properties of *Pseudomonas anguilliseptica* in agglutination. *Bull. Japan. Soc. Sci. Fish.*, **47**, 699–703.

NAKAI, T., K. MUROGA, and H. WAKABAYASHI (1982): An immunoelectrophoretic analysis of *Pseudomonas anguilliseptica* antigens. *Bull. Japan. Soc. Sci. Fish.*, **48**, 363–367.

ROGERS, D. E. (1960): Host mechanisms which act to remove bacteria from the blood stream. *Bact. Rev.*, **24**, 50–66.

SHIMIZU, T. (1969): Studies on pathogenic properties of *Aeromonas liquefaciens*—I. Production of toxic substance to eel. *Bull. Japan. Soc. Sci.*

Fish., **35**, 55–66.

STEWART, D. J., K. WOLDEMARIAM, G. DEAR, and F. M. MOCHABA (1983): An outbreak of 'Sekiten-byo' among cultured European eels, *Anguilla anguilla* L., in Scotland. *J. Fish Diseases*, **6**, 75–76.

WAKABAYASHI, H. and S. EGUSA (1972): Characteristics of a *Pseudomonas* sp. from an epizootic of pond-cultured eels (*Anguilla japonica*). *Bull. Japan. Soc. Sci. Fish.*, **38**, 577–587.