

Experimental *Streptococcus iniae* Infection in Japanese Flounder *Paralichthys olivaceus*

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(Received December 13, 2000)

ABSTRACT—The capability of *Streptococcus iniae* to cause disease in Japanese flounder was investigated by oral and bath (30 min) challenges with low, medium and high inoculation doses. Only with the highest inoculation dose, 9.9×10^7 CFU/100 g body weight, the oral challenge induced deaths. In the bath challenge deaths were induced even at the lowest inoculation dose, 2.9×10^3 CFU/mL water. Hemorrhagic lesions on the fins were observed in dead fish challenged by both methods. It is suspected that *S. iniae* entered through the body surface such as abrasive sites of the fins to cause disease.

Key words: *Streptococcus iniae*, *Paralichthys olivaceus*, oral challenge, bath challenge, Japanese flounder

Streptococcus iniae is one of the principle causative agents of streptococcosis in many cultured fish. The infection routes of *S. iniae* have been suggested through experimental infections. Through the digestive tract as well as directly from the water *S. iniae* can cause disease in blue tilapia *Oreochromis aureus*¹⁾. The olfactory organ has been suggested as a possible route of *S. iniae* infection for hybrid striped bass *Morone chrysops* × *M. saxatilis* and Nile tilapia *O. niloticus*²⁾. *Lactococcus garvieae*, a pathogen of fish streptococcosis, has been suggested to infect yellowtail *Seriola quinqueradiata* through the digestive tract³⁾.

Streptococcosis in Japanese flounder *Paralichthys olivaceus*, an important flatfish species in mariculture in Japan, often occurs during the period of high water temperature⁴⁾. Infection routes of *S. iniae* in flounder are not clear. Elucidation of possible routes of the infection will be helpful to establish more effective fish health management. The purpose of the present work is to investigate the capability of *S. iniae* to cause disease by oral and bath challenge with three inoculation doses.

Materials and Methods

Flounder (average weight 101 ± 16 g) were stocked into 200-L tanks for challenge groups of 20 fish each and 30-L tanks for control groups of 5 fish each one day before challenge. Each tank was supplied with constant aeration and continuous water flow at 6 L/min. Water temperature ranged from 25 to 26°C and fish received no food during the experiment.

S. iniae NUF631, originally isolated from a diseased flounder, was cultured on Todd-Hewitt (TH) agar plates at 28°C, harvested 18 h after incubation by washing off the plates with 0.1M phosphate-buffered saline (PBS), pH 7.2, to make a bacterial suspension. The experiment was carried out with low, medium and high inoculation doses as described below.

For oral challenge, viable count of the bacterial suspension was confirmed by TH agar plate count (duplicate). Commercial pellet feed ground thoroughly by a grinder was mixed with an appropriate volume of the bacterial suspension at a ratio of 1(wt) : 2.5(v) to produce an injectable slurry. Each fish was anesthetized with tricaine methane sulfonate and received the slurry at a volume of 2% body weight by intubation. The intubation was performed with a 10-mL syringe attached with a plastic catheter that was inserted to the stomach of experimental fish. Inoculation doses were 9.9×10^3 (low), $\times 10^5$ (medium) and $\times 10^7$ (high) colony forming units (CFU)/100 g body weight. The fish in control group received the luxury made with PBS only.

For bath challenge, water in the tanks was drained until the remaining was 10 L and then the bacterial suspension was poured in. Viable count of *S. iniae* in the water was confirmed by TAOA agar plate count (duplicate). TAOA agar, a selective medium for *S. iniae*, was prepared according to the descriptions in our published work⁵⁾. Fish were exposed to the bacterium for 30 min. At the end of exposing time, the tanks were filled in with water to reach the initial water level for 30 min, and then water change was commenced again. Inoculation doses were 2.9×10^3 (low), $\times 10^5$ (medium) and $\times 10^7$ (high) CFU/mL water. No bacteria were poured into the tank of control group.

Number of survivors was monitored daily for a 14 day period after challenge. Dead fish were taken off and examined for gross lesions both externally and internally. Their brain and kidney were cultured on TH agar and then the grown colonies, if present, were subjected to agglutination tests with rabbit anti-*S. iniae* NUF631 serum to confirm *S. iniae* infection. Survivors at the end of the experiment were also examined in the same way as dead fish.

Results and Discussion

No deaths were observed in the control groups and

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the oral challenge groups with the low and medium inoculation doses. In the other challenge groups, deaths were observed. Death in these groups began at day 2 and the majority of deaths occurred between 2 and 7 days after challenge except for those observed in the bath challenge group with the low inoculation dose. The initiation of deaths in this group occurred later, at day 5 (Figs. 1 and 2). *S. iniae* was recovered from all dead fish and one survivor in the bath challenge group with the low inoculation dose.

Gross examinations of the early deaths showed, externally, small hemorrhagic lesions on the fins, particularly the dorsal and pectoral fins, and, less frequently, on the skin. Internally, mild swelling of the liver, spleen and kidney was observed. For those of the later deaths,

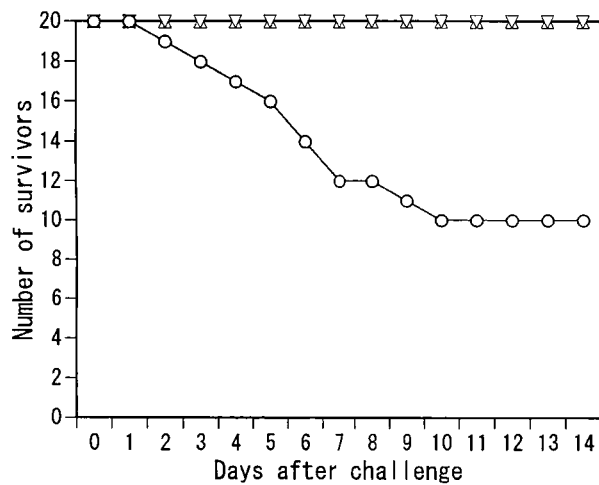


Fig. 1. Changes in number of survivors after oral challenge with *S. iniae* NUF631. Inoculation dose (CFU/100 g body weight); 9.9×10^7 (○), 9.9×10^5 (▽), 9.9×10^3 (△).

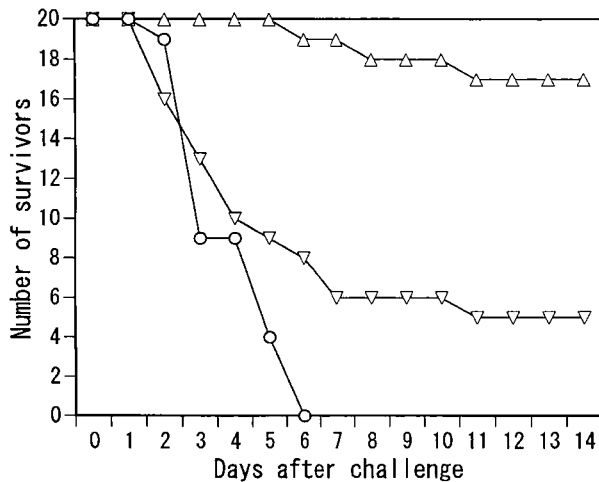


Fig. 2. Changes in number of survivors after bath challenge with *S. iniae* NUF631. Inoculation dose (CFU/mL water); 2.9×10^7 (○), 2.9×10^5 (▽), 2.9×10^3 (△).

pathological lesions were more obvious. Hemorrhages of fin were severe and extended to the proximal margin. Congestion of the liver, spleen and kidney resulting in severe swelling and darkness in color of these organs were observed. Some dead fish showed typical lesions of the disease such as clouding cornea, internal hemorrhagic and exophthalmic eyes, ascites resulting in abdominal swelling, and pale liver, spleen and kidney. Gastric and intestinal lesions were not observed in any dead fish. These observations were common in the fish challenged by either challenge method.

Although the oral challenge was reported to produce *S. iniae* infection¹⁾, the capability of the bacterium to enter through the mucosal epithelia of the stomach and/or intestine has not been proved. If *S. iniae* possesses this capability indeed, it requires, as the present result has shown, a high number of viable cells to cause disease. It may be such a case in practical culture of flounder that flounder ingests decayed visceral organs or body of infected fish. Otherwise deaths in the oral challenge group employed with the high inoculation dose may due to the infection of *S. iniae* excreted to the tank along with feces, because hemorrhagic lesions on the fins of dead fish were observed like bath-challenged fish and relatively low number of viable cells was enough to cause disease in the bath challenge groups.

In the bath challenge experiment deaths were induced even at the lowest inoculation dose. This result implies even though fish may ingest *S. iniae*-containing water during the exposing period, it is unlikely to occur the oral route infection. It has been suggested that transmission of streptococcosis caused by *S. iniae* from fish to fish could occur via direct contact and abrasion⁶⁾. Basing on the observation of hemorrhagic lesions on the fins of dead fish, it seems reasonable to suspect that *S. iniae* entered through the body surface such as abrasive sites of the fins.

The result of this work showed that *S. iniae* infection could be induced by oral and bath challenge, though bath challenge induced disease more easily than oral challenge. Bacteriological and histopathological studies are needed to clarify the exact sites of entry and the course of infection.

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