Short communication

Skin Injuries Contribute to Nocardiosis in Japanese Amberjack *Seriola quinqueradiata*

Yasuyuki Miyoshi¹, Yutaka Fukuda²
and Kazuo Ogawa^{3*}

¹Oita Prefecture Southern Region Bureau,
Oita 876-0813, Japan

²Fisheries Research Division, Oita Prefectural Agriculture,
Forestry and Fisheries Research Center,
Oita 879-2602, Japan

³Meguro Parasitological Museum,
Tokyo 153-0064, Japan

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ABSTRACT—We examined how mechanical damages to the skin and the infestation of the monogenean *Benedenia seriolae* on the skin affected *Nocardia seriolae* infection in Japanese amberjack *Seriola quinqueradiata*. After the skin was inflicted damages or after *B. seriolae* was dewormed with freshwater bathing, amberjacks were challenged with 1.0×10^3 CFU/mL *N. seriolae* by immersion. Survival rate was significantly lower in the skin-damaged group and the skin fluke-infested group than in their respective control groups. The present study suggests that skin injuries and *B. seriolae* infection contribute to the *N. seriolae* infection in Japanese amberjack.

Key words: *Seriola quinqueradiata*, *Nocardia seriolae*, skin injuries, *Benedenia seriolae*, skin fluke

Nocardia seriolae, a Gram-positive, weakly acid-fast, filamentous bacterium, is the causative agent of nocardiosis of cultured amberjacks *Seriola* spp. in Japan (Kudo *et al.*, 1988). This infectious disease was first noticed in ca. 1967 (Kariya *et al.*, 1968) and is still causing economic damages to the aquaculture industry. *N. seriolae* is sensitive to several antimicrobial agents *in vitro* (Itano and Kawakami, 2002), which, however, show a low killing effect *in vivo* on the bacterium in the nodules formed in the viscera of infected fish. Currently, several sulfonamides like sulfamonomethoxine are approved to be used as therapeutic agents of nocardiosis, but it is practically difficult to diagnose fish in an early stage of infection before the nodule formation (Itano *et al.*, 2008). Development of vaccines against nocardiosis is

expected, which is currently in experimental conditions (Kato *et al.*, 2014). For the moment, control of this disease largely depends on the culture techniques to avoid infection.

It has long been suspected that Japanese amberjack (=yellowtail) *Seriola quinqueradiata* with skin injuries are vulnerable to *N. seriolae* infection (Kusuda and Nakagawa, 1978). Matsuzato (1978) speculated that injuries of the skin and the gills induced by the monogeneans *Benedenia seriolae* and *Heteraxine heterocerca*, respectively, can form a portal of entry by *N. seriolae*. However, no experimental evidence has been presented on the involvement of injuries and monogenean infestations in the *N. seriolae* infection of amberjacks. In this paper, we examined experimentally 1) if physical injuries of the skin induce *N. seriolae* infection of Japanese amberjack, and 2) how the infestation of *B. seriolae*, a ubiquitous skin parasite among amberjacks cultured in net cages (Ogawa and Shirakashi, 2017), contributes to the progression of nocardiosis.

Materials and Methods

Fish used in the experiments

0-year-old wild-caught seedlings of Japanese amberjack *Seriola quinqueradiata* were used for the experiments below. Prior to the experiments, during preliminary maintenance in net cages, they got infested by the skin fluke *Benedenia seriolae*, which was dewormed by freshwater bathing every 2 weeks. No infestation with another monogenean *Heteraxine heterocerca* was noticed.

Trial 1 (Physical injury)

Randomly selected amberjack (100.2 g in mean body weight, $n = 10$ for each group) were used for Trial 1. For the experimentally injured group, after anesthetization using eugenol (FA100, DS Pharma Animal Health Co., Ltd), tube needles for BCG inoculation (Japan BCG Laboratory), a tube apparatus equipped with nine short needles inside, were applied once on both left and right surfaces of the posterior head and central trunk parts (= four body parts with nine needles for each fish). No treatment was done to control fish except for anesthetization.

For infection experiments, *Nocardia seriolae* (strain number 024013) isolated from a Japanese amberjack cultured in Tsukumi Bay, Oita Prefecture in 2002 was used. The bacterium was inoculated into Brain Heart Infusion Broth (Difco) with 10 glass beads (3 mm in diameter) and incubated with rotating at 150 rpm at 25°C on a rotary shaker (Miyamoto Riken Co., Ltd) for 6 days. Subsequently, the bacterium was harvested by centrifuge, washed once with sterile PBS and mixed with seawater at the concentration of 1.0×10^3 CFU/mL or 1.0×10^4 CFU/mL. Fish of the experiment and control groups

* Corresponding author
E-mail: ogawak@kiseichu.org

were exposed to the aerated seawater containing *N. seriolae* at the above concentrations for 10 min. After challenge, fish of each group ($n = 10$) were kept in a 500 L seawater at 25°C in a recirculated water system for 3 weeks without feeding. All fish, immediately after death and, in case of survivors, at the end of the experiment, were subjected to biopsy, and bacterial isolation was conducted from the kidney onto the Mycobacteria 7H11 Agar (Difco, with OADC enrichment, without glycerol) plates, which were incubated at 25°C for 5 days.

Trial 2 (Injury by skin fluke infestation)

Japanese amberjack infested with *B. seriolae* were dewormed with freshwater bathing and fed with commercial EP pellets for 20 days in a floating sea cage to be exposed to oncomiracidia of the skin fluke or in an aquarium with running sand-filtered seawater to be kept free from oncomiracidia. During the rearing period, water temperature was 25.2°C in the cage and 24.6°C in the aquarium. At the end of the rearing period, the fish was 205.5 g in mean body weight and mean intensity ($\pm$ SD) of *B. seriolae* on the cage fish (infested group) was 9.8 ± 2.4 (no. worms/ fish), whereas there was no skin fluke on the aquarium fish (un-infested group).

Each fish group was divided into two sub-groups, which were kept either in aerated seawater or to freshwater for 3 min, the latter treatment being enough to remove skin flukes, if infested, from fish. Seawater containing 1.0×10^3 CFU/mL *N. seriolae* was prepared as in Trial 1, and the four groups of fish were challenged with *N. seriolae* by immersion for 10 min. Subsequently, fish of each group ($n = 10$) were randomly selected and kept in a 450 L seawater aquarium in a recirculated water system for 3 weeks without feeding (mean water temperature: 27.4°C). Besides, some of *B. seriolae* infested and *N. seriolae* un-challenged fish were reared in the same condition as the challenged groups to compare the effect of the skin fluke infestation on the fish exposed to *N. seriolae* with those without exposure. After the experiment, All fish, immediately after death and, in case of survivors, at the end of the experiment, were subjected to biopsy, and bacterial isolation was conducted from the kidney or spleen with Mycobacteria 7H11 Agar and 1.0% NaCl-added Tryptone-Soya Agar (Nissui).

Results

Trial 1 (Physical injury)

Results of the infection experiments were shown in Fig. 1. When challenged by immersion with *Nocardia seriolae* at 1.0×10^3 CFU/mL, the survival rate at 3 weeks post-exposure (PE) was 10% in the injured group vs. 60% in the uninjured control, the ratio being significantly different from each other by Kaplan-Meier analysis and log-rank test ($p = 0.02$). When challenged at

1.0×10^4 CFU/mL, the survival rate was 0% by day 19 PE in the injured group vs. 40% at 3 weeks PE, being not different from each other. *N. seriolae* was isolated from all the dead fish immersed in both doses. *N. seriolae* was isolated from all survivors in the injured group, whereas it was recovered from 60% and 80% of the survivors in the control challenged at 1.0×10^3 CFU/mL and at 1.0×10^4 CFU/mL, respectively.

Trial 2 (Injury by skin fluke infestation)

Results of the challenge experiments to fish without freshwater bathing were shown in Fig. 2. All fish in the *Benedenia seriolae* infested group were dead by day 14 PE, whereas the survival rate in the un-infested group was 80% at 3 weeks PE, the ratio being significantly different from each other (Kaplan-Meier analysis and log-rank test, $p = 0.0003$). *N. seriolae* was not isolated from 20% of the dead fish in the infested group. On the other hand, in the un-infested group, *N. seriolae* was isolated from all dead fish and from 62.5% of the survivors. In the fish unchallenged with *N. seriolae*, all fish were dead by day 17. They were heavily infested with skin flukes, but no pathogenic bacteria including *N. seriolae*, were isolated.

Fig. 1. Survivals of Japanese amberjack (●: injured fish, ○: uninjured fish) challenged with 1.0×10^3 CFU/mL *Nocardia seriolae* by immersion and amberjack (▲: injured fish, △: uninjured fish) with 1.0×10^4 CFU/mL by immersion.

Fig. 2. Survivals of Japanese amberjack (●: skin fluke infested, ○: un-infested) challenged with 1.0×10^3 CFU/mL *Nocardia seriolae* by immersion. ▲: skin fluke infested and un-challenged amberjack.

Fig. 3. Survivals of Japanese amberjack challenged with 1.0×10^3 CFU/mL *Nocardia seriolae* by immersion (●: skin fluke infested fish after dewormed by freshwater bathing, ○: un-infested fish after freshwater bathing).

Results of the challenge experiments to fish treated with freshwater bathing were shown in Fig. 3. The survival rate was 10% at 3 weeks PE in the infested group vs. 90% in the un-infested group, the ratio being significantly different from each other (Kaplan-Meier analysis and log-rank test, $p = 0.0005$). No skin fluke infestation was observed in both groups. *N. seriolae* was isolated from all dead fish, and 100% and 44.4% from survivors of the infested and un-infested group, respectively.

Discussion

Effects of skin damages on the susceptibility to bacterial infections of injured fish have been evaluated experimentally. For example, channel catfish *Ictalurus punctatus* with skin injuries were fatally infected with *Flavobacterium columnare* and virulent *Aeromonas hydrophila* (Bader *et al.*, 2006; Zhang *et al.*, 2016). In this study, we aimed to evaluate effects of skin injuries on the vulnerability of Japanese amberjack to *Nocardia seriolae* infection by immersion. Itano *et al.* (2006) calculated the LD_{50} value of *N. seriolae* of Japanese amberjack (60.8 g in mean body weight) as 1.2×10^4 CFU/mL by immersion. Based on this, we challenged injured Japanese amberjack at 1.0×10^4 CFU/mL, in which mortality was higher than expected and no difference was observed between the injured and uninjured fish. In the following experiment with a lower dose (1.0×10^3 CFU/mL) by immersion, a significant difference in the survival rate was observed between the experiment and control group, and the injured group had a higher rate of *N. seriolae* isolation than the uninjured group. A low dose challenge may be suitable for the evaluation of the injury effect. The present results suggest that skin injuries induce *N. seriolae* infection of Japanese amberjack.

Many authors reported that skin parasites promoted bacterial infections of fish. Shimura *et al.* (1983) experimentally demonstrated that masu salmon *Oncorhynchus masou* infested with the parasitic crustacean *Argulus coregoni* on the skin were more susceptible to *Aeromonas salmonicida* infection than uninfested

control. Xu *et al.* (2007) showed that Nile tilapia *Oreochromis niloticus* infested with the monogenean *Gyrodactylus niloticus* had a higher mortality rate due to streptococcosis induced by immersion of *Streptococcus iniae* than the monogenean-free fish. They suggested that injuries on the external surfaces of Nile tilapia caused by *G. niloticus* infestation created portals of entry for bacterial invasion.

Benedenia seriolae is a serious pathogen of cultured Japanese amberjack, causing skin injuries due to feeding of host epithelial tissues and sucking actions by the opisthohaptor and a pair of preoral suckers (Ogawa and Shirakashi, 2017). Infested fish in the cage rub against the net to get rid of the parasite, which may lead to more serious damages of the skin like hemorrhagic ulceration (Hoshina, 1968; Egusa, 1983). It has long been hypothesized that skin injuries caused by *B. seriolae* provide portals of entry for microbial invasion (Hoshina, 1968; Egusa, 1983). However, this hypothesis has never been tested experimentally.

In this study, we followed the disease progression of *B. seriolae*-infested Japanese amberjack after immersion challenge with *Nocardia seriolae*. As a result, all skin fluke-infested fish were dead, with the survival rate being significantly lower than those without skin flukes. However, *N. seriolae* was not isolated from some of the dead fish after the immersion challenge, and all skin fluke-infested fish without immersion were dead. From these results it could not be denied that some of the skin fluke-infested and *N. seriolae* immersed fish may have died of *B. seriolae* infestation.

As a next step, we examined the effect of skin injuries due to *B. seriolae* infestation on *N. seriolae* infection after deworming flukes from Japanese amberjack with freshwater bathing. The experiments showed a significantly lower survival rate among dewormed fish than un-infested control. Besides, the rate of *N. seriolae* recovery from the survivors was significantly higher than the un-infested control. From all these, it was suggested that Japanese amberjack with skin injuries due to *B. seriolae* infestation are more susceptible to *N. seriolae* infection.

Kusuda and Nakagawa (1978) pointed out that Japanese amberjack were constantly exposed to *N. seriolae* in nutrient-rich seawater at some culture farms, as the bacterium could survive for months in polluted seawater. They suspected that *N. seriolae* could have more chances to invade fish through skin injuries, causing nocardiosis in polluted water. The present results confirm that skin injuries are a factor responsible for the *N. seriolae* infection of amberjack and suggest that *B. seriolae*, a ubiquitous parasite at amberjack farms, is involved in nocardiosis and the progression of the disease. It is thus important to avoid mechanical injuries on the skin and to control the skin fluke infestation in the management of amberjack culture. Currently, efficient

control of nocardiosis among cultured Japanese amberjack is quite limited, as chemotherapy using sulfonamides is the only choice and effective vaccines are still under development. Prophylactic measures, then, should be emphasized in the future study. The present results suggest that it is important to keep the skin intact to prevent nocardiosis. In this respect, nutritional studies will be required for the development of a new prophylactic measure to enhance the defense mechanism of the skin and accelerate the recovery from injuries.

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