Comparison of Immersion-vaccination Against Gliding Bacterial Disease in Red Sea Bream *Pagrus major* and Japanese Flounder *Paralichthys olivaceus*

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**Abstract:** Two strains, R2 from red sea bream and GF0609 from Japanese flounder, of *Tenacibaculum maritimum*, causative agents of gliding bacterial disease, were grown on modified cytophaga agar medium containing 70% seawater for 48 h at 25\(^\circ\)C and were inactivated with 1.5% (v/v) formalin-PBS for 48 h at 4\(^\circ\)C (FKC-R2 and FKC-GF). To evaluate efficacies of the FKCs in red sea bream and Japanese flounder, fishes were immersion-vaccinated with each FKC (20\(\mu\)g wet weight/ml) for 20 min, and were experimentally challenged with the R2 and/or GF0609 strains at 10 days post vaccination. In the challenge by R2 strain, survival rates of vaccinated red sea bream with FKC-R2 and FKC-GF were 80% (RPS: 75%) and 40% (RPS: 25%), with a significant difference from that of control (\(P < 0.05\)). No vaccination efficacy of the FKCs was observed in Japanese flounder challenged by the GF0609. Moreover, susceptibilities of red sea bream against GF0609 and Japanese flounder against R2 were revealed to be low.

**Key words:** Gliding bacterial disease; Immersion-vaccination; Japanese flounder; Red sea bream

Gliding bacterial disease was first described in red sea bream *Pagrus major* and black sea bream *Acanthopagrus schlegeli* in Japan in the 1970s (Masumura and Wakabayashi 1977). At present, the majority of cultured marine fish species in Japan, especially red sea bream and Japanese flounder, are affected by this disease (Baxa et al. 1986, 1987; Wakabayashi et al. 1986; Bernerdet 1998). The occurrence of this disease has been reported from Europe (MacVicar and White 1979; Alsin and Blanch 1993; Bernerdet et al. 1990, 1994; Bernerdet 1998), Australia (Soltani and Burke 1994; Handlinger et al. 1997) and the US (Chen et al. 1995) in recent years. The pathogen of gliding bacterial disease, *Tenacibaculum maritimum* (syn. *Flexibacter maritimus*) (Wakabayashi et al. 1986, Suzuki et al. 2001), has strong proteolytic activity; hence, diseased fish exhibits fin erosion and necrotic ulcers of the skin and muscle (Masumura et al. 1977; Baxa et al. 1986; Alsin et al. 1993; Bernerdet 1998).

Immersion-treatment with antibacterial agents, such as sodium nifrustyrenate, is effective against the disease (Baxa et al. 1988, Bernerdet 1998), but in Japan its use is currently restricted to Japanese flounder *Paralichthys olivaceus* juveniles. The permitted antibacterial agents are, therefore, administered orally to treat other cultured fish species. However, it is difficult to achieve the desired effect owing to the poor appetite of the affected fish.

It was reported that resistance was not acquired in Dover sole *Solea solea* that survived the disease (MacVicar and White 1979). A vaccine developed for turbot (*Scophthalmus maximus*) has recently been patented in Spain (Toranzo et al. 2005). In addition, enhancement of the immune response of sea bass *Dicentrarchus labrax* to *T. maritimum* (Salati et al. 2005) and the efficacy of immersion-vaccination in red sea bream (Kato et al. 2006) have also been reported.
Nevertheless, it is unclear whether the vaccination against the disease is effective in other cultured marine fish species, especially Japanese flounder, which is affected severely.

The aim of this study was to evaluate the effect of immersion-vaccination on the disease in Japanese flounder. In addition, the efficacies of two types of formalin-killed cells (FKC), prepared from isolates from red sea bream and Japanese flounder, were compared by challenging immersion-vaccinated red sea bream and Japanese flounder with both strains.

**Material and Methods**

**Experimental fishes**

Red sea bream \((n=60, \text{mean weight 30.7 g})\) and Japanese flounder \((n=60, \text{mean weight 63.6 g})\), obtained from cultured brood stock at the Fisheries Laboratory of Kinki University, Shirahama Station, were used. Mean water temperature were at 21.7 and 18.9°C over the experimental period for red sea bream and Japanese flounder, respectively.

**Experimental strains of bacteria**

Two strains of *T. maritimum*, R2 (=ATCC 43398) and GF0609, were used. The R2 was originally isolated from a red sea bream at Hiroshima Prefecture in 1977 (Wakabayashi et al. 1986) and kindly provided by Dr. Kazuo Ogawa of Tokyo University. The GF0609 was isolated from a cultured Japanese flounder at Fisheries Laboratory of Kinki University, Shirahama Station, in 2003.

**Preparation of FKC**

Two types of FKC, FKC-R2 and FKC-GF, were prepared from strains R2 and GF0609 respectively. Each strain was cultured with modified cytophaga agar medium containing 70% seawater (MCA70) for 48 h at 25°C. The cells were collected by scraping and suspended in 1.5% (v/v) formalin-PBS for 48 h at 4°C. The preparations were kept at 4°C until use as vaccine.

**Vaccination**

The experimental design of each fish species was consisted of two sets of three groups. Each group had 10 fish, which were kept in 200-l tanks supplied with running water. In each set, two groups were vaccinated with FKC-R2 or FKC-GF, while the other group received no treatment. Vaccination was performed by immersion in FKC suspensions in seawater \(20\mu g \text{ wet wt./ml}\) for 20 min.

**Experimental challenge**

Ten days after vaccination, each set of red sea bream and Japanese flounder was challenged with R2 or GF0609. Stamp injection was performed using an injector, consisting of a hollow plastic cylinder with nine needles, designed for human BCG vaccination. The body surface of the experimental fish was pressed with the cylinder after soaking the tip of the needles in the bacterial suspensions for a few seconds. The R2 and GF0609 were grown on MCA70 for 24 h at 25°C, and suspended in PBS for stamp injection; doses of R2 were \(2.2 \times 10^{10} \text{ CFU/ml}\) for red sea bream and \(6.6 \times 10^{10} \text{ CFU/ml}\) for Japanese flounder, and those of GF0609 were \(3.0 \times 10^{10} \text{ CFU/ml}\) for red sea bream and \(6.1 \times 10^{10} \text{ CFU/ml}\) for Japanese flounder, respectively. Dead fish were counted daily until 7 days post-challenge. Affected areas of the body surface were observed under an optical microscope. Affected site of the body surface, liver and kidney were smeared onto MCA70 in an attempt to isolate the pathogen.

**Result**

**Red sea bream**

Changes in survival rate after challenge with R2 are shown in Fig. 1. Survival rates at the end of the experiment in the FKC-R2, FKC-GF and control groups were 80, 40 and 20%, respectively. Relative percent survivals (RPS) for the FKC-R2 and FKC-GF groups were 75 and 25%, respectively. Higher survival rates were observed in the vaccinated groups, and a significant difference \((\chi^2 \text{ test}, P<0.05)\) was shown between the FKC-R2 and control groups. Changes in survival rate after challenge with GF0609 are shown in Fig. 2. Survival rates in the FKC-R2, FKC-GF and control groups were 100, 100 and 90%, respectively.
Fig. 1. Changes in the survival rate of immersion-vaccinated red sea bream after challenge with R2 strain. (□) Vaccinated with FKC-R2, (△) vaccinated with FKC-GF, (○) control.

Fig. 2. Changes in the survival rate of immersion-vaccinated red sea bream after challenge with GF0609 strain. (□) Vaccinated with FKC-R2, (△) vaccinated with FKC-GF, (○) control.

respectively, indicating that GF0609, isolated from Japanese flounder, has low virulence to red sea bream. Ulcerative lesion was observed on the stamped sites of all the dead fish and some survivors. Gliding bacterial cells were observed on the affected areas under an optical microscope, but no Tenacibaculum colony was recovered on MCA70 from affected areas, even though one type of Vibrio-like colonies were isolated. No bacterial colonies were obtained from the liver and kidney.

Japanese flounder

Changes in survival rate after challenge with R2 are shown in Fig. 3. Survival rates in the FKC-R2, FKC-GF and control groups were 80, 100 and 90%, respectively. This result suggests that the R2 strain is not virulent to Japanese flounder. Changes in survival rates after challenge with GF0609 are shown in Fig. 4. Survival rates in the FKC-R2, FKC-GF and control groups were 0, 0 and 10%, respectively. No significant difference (χ² test, P<0.05) was shown between all the experimental groups. Ulcerative lesion on the stamped sites was observed similar to those in red sea bream and gliding bacterial cells were observed on the affected areas. Only one type of Vibrio-like colonies was isolated from affected areas, instead of T. maritimum. No bacterial colonies were obtained from the liver and kidney.

Discussion

We have already reported the high efficacy of FKC vaccine in red sea bream. Therefore, this study have evaluated the vaccine in Japanese flounder. In addition, the efficacies of FKC
derived from red sea bream and Japanese flounder isolates were compared in both fish species. After experimental challenges, gliding bacterial cells were observed on the affected areas but no *Tenacibaculum* colonies were recovered from dead fish. It is known that the growth of *T. maritimum* is inhibited by other bacteria (Pazos et al. 1996). The *Vibrio*-like bacteria isolated in this study may have prevented the growth of *T. maritimum*.

Mortalities of red sea bream by GF0609 and Japanese flounder by R2 showed low mortalities. This suggests that GF0609 and R2 included some differences in host-specific virulence factors.

In red sea bream challenged with R2, high RPS (75%) was obtained in the FKC-R2 group. This result confirms that immersion-vaccination is effective against *T. maritimum* infection in red sea bream. However, the FKC-GF group showed low RPS (25%), and it was thus considered that there is a difference in virulence factor between R2 and GF0609. Currently, three serotypes have been reported in *T. maritimum* (Avendaño-Herrera et al. 2004; 2005); therefore, extensive investigation is required on the relationship of antigenicity and pathogenicity between serotypes.

In Japanese flounder challenged with GF0609, there were no significant differences in survival rates among both FKC groups and the control. Japanese flounder may have slow reaction in the immune response because ten days period may be short to raise enough immune response and higher susceptibility to *T. maritimum* infection rather than red sea bream. The kinetics of immune-response in Japanese flounder might be necessary to confirm the effectiveness of immersion vaccination and make up effective plan for the immunization of this fish.

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**Reference**


Vaccination Against Gliding Bacterial Disease


マダイおよびヒラメにおける滑走細菌症に対する浸漬ワクチンの効果

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Tenacibaculum maritimum のマダイ分離株 R2 およびヒラメ分離株 GF0609 を用いてホルマリン不活化菌体（それぞれ FKC-R2, FKC-GF）を作製し、マダイおよびヒラメを供試魚としてワクチンとしての効果を比較検討した。FKC は70%海水改質サイトフィーダー型添加地で25℃で48時間培養した菌体を1.5%ホルマリン PBS で4℃で48時間不活化することで作製した。ワクチン処理は FKC の浸漬（20μg/ml, 20 min）によって行った。免疫10日に後 R2 および GF0609 の生菌を用いて攻撃を行い、ワクチンの効果を検討した。R2 で攻撃したマダイでは、両 FKC 免疫区で対照区より高い生残率が得られたが、ヒラメではワクチンの効果が認められなかった。また、マダイは GF0609 による攻撃に対して、ヒラメは R2 による攻撃に対してそれぞれ感受性が低かった。