Detection of Japanese Flounder Antibody against Fimbrial Major Protein of *Edwardsiella tarda*

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ABSTRACT—A recombinant fimbrial protein of *Edwardsiella tarda* was designed to detect the specific antibody in Japanese flounder *Paralichthys olivaceus*. The specific antibody was detected from the fish intraperitoneally injected with *E. tarda* expressing the fimbria on LB agar containing 3% NaCl, but not from those injected with *E. tarda* without the fimbria, *Streptococcus iniae*, *S. parauberis* or viral hemorrhagic septicemia virus. In a flounder farm suffered with edwardsiellosis, the antibody-positive fish increased in ration with the occurrence of mortality. These results suggested that *E. tarda* with the fimbria was important for epidemics of edwardsiellosis at flounder farms.

Key words: *Edwardsiella tarda*, fimbrial protein, immunodot-blot test, *Paralichthys olivaceus*

Edwardsiella tarda is a facultative anaerobic enterobacterium¹⁾ that is known to be the causative agent of edwardsiellosis in freshwater and marine fish^{2–4)}. In Japan, the Japanese flounder *Paralichthys olivaceus* farming industry has suffered particularly serious damage from this disease⁵⁾. Many studies have investigated the virulence properties of *E. tarda*, in particular, the mechanisms for hemolysis⁶⁾ and anti-phagocytic killing^{7,8)}. As another virulence factor of *E. tarda*, Yasunobu *et al.*⁹⁾ reported that hemagglutinating activity and infectivity of *E. tarda* to Japanese flounder are enhanced by the presence of NaCl. In addition,

hemagglutinating activity was proved to be correlated with the adherence of *E. tarda* to HEp-2 cells¹⁰⁾. Fimbria, which contains a 19.3 kDa major subunit, reportedly causes hemagglutination¹¹⁾, and the genes coding fimbria are conserved among fish-pathogenic *E. tarda* strains¹²⁾. Therefore, fimbria is considered to be an adhesin essential for *E. tarda* infection in marine fish. To clarify the presence of *E. tarda* having fimbria in Japanese flounder at the farm, this study investigated the production of antibody against fimbria in Japanese flounder by immunodetection with a recombinant protein of fimbrial major subunit.

Template DNA for the recombinant protein was prepared from the *E. tarda* strain NUF806 by PCR with primers, 5'-GCTAACGGTAAAGTTGAGTTTACG-3' and 5'-TTTATATTGATGGTGAACGGGC-3', which were designed at nucleotide positions 67–90 and 509–531, respectively, on the fimbrial major subunit gene *effA* of *E. tarda* (GenBank accession no. AB100170). PCR was performed with Phusion High-Fidelity DNA Polymerase (Finnzymes) according to the manufacturer's protocol. The PCR product was cloned into a His-tagged prokaryotic expression vector pET30 (Novagen) and then transduced into *Escherichia coli* BL21 (DE3) (Nippon Gene). The recombinant protein was biosynthesized by cultivation using Overnight Express Instant TB Medium (Merck) at 25°C for 72 h, then isolated with TALON CellThru Resin (Clontech) according to the manufacturer's protocol and stored in phosphate-buffered saline (PBS) containing 0.02% sodium azide until use (100 ng/μL). The purified recombinant protein was observed by SDS-PAGE according to Laemmli¹³⁾ (Fig. 1). This recombinant protein showed the antigenicity against a rabbit antiserum for the fimbrial major subunit protein in the immunoblot-

Fig. 1. SDS-PAGE of purified recombinant *E. tarda* fimbrial major subunit. Lane M: molecular weight markers; lane 1: the recombinant protein.

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ting method of Sakai *et al.*¹¹⁾.

Kim *et al.*¹⁴⁾ reported that ELISA for antibody detection in Japanese flounder has low reproducibility due to non-specific adsorption of the immunoglobulin M (IgM) to blocking reagents on the ELISA plate wells. In the present study, the immuno-dot-blot test was used to detect the antibody because the result was highly reproducible. The recombinant suspension (1 μ L) was transferred onto 0.22- μ m nitrocellulose membrane (Nitro Bind, GE Osmonics). The membrane was blocked with 3% non-fat dry milk (Bio-Rad Laboratories) for 1 h and then immersed in tenfold diluted Japanese flounder serum with PBS containing 0.02% Tween 20 (T-PBS) for 1 h. After washing 4 times with T-PBS, immunochemical staining was carried out with a mouse monoclonal antibody against the IgM antibody of Japanese flounder¹⁵⁾ by use of Immun-Blot Alkaline Phosphatase Assay Kit (Bio-Rad Laboratories) according to the manufacturer's protocol.

Specificity of the recombinant protein in the immuno-dot-blot test was confirmed using sera from Japanese flounder (approximate body weight: 700 g) experimentally infected with *E. tarda*, *Streptococcus iniae*, *S. parauberis* or viral hemorrhagic septicemia virus (VHSV), all of which are major pathogens of Japanese flounder^{16–18)}. *E. tarda* NUF806 was cultured on Luria-Bertoni agar (Becton Dickinson, 0.5% NaCl agar) without or with added NaCl at 2.5% (3% final concentration; 3% NaCl agar) at 27°C for 48 h. Production of the fimbrial major subunit protein was observed by the immunoblotting method of Sakai *et al.*¹¹⁾. *S. iniae* NUF812 and *S. parauberis* FPC1060 were cultured on Todd-Hewitt agar (Becton Dickinson) at 27°C for 24 h. The VHSV strain JF00Ehi1 was cultured according to the method of Ito *et al.*¹⁹⁾. In each challenge test, fish ($n = 30$) were injected intraperitoneally with *E. tarda* NUF806 at 5.2×10^4 CFU/fish, *S. iniae* NUF812 at 8.5×10^5 CFU/fish, *S. parauberis* FPC1060 at 1.5×10^6 CFU/fish and VHSV JF00Ehi1 at $10^{5.3}$ TCID₅₀/fish. Fish of the bacterial-challenged or control group were kept at $20 \pm 1^\circ\text{C}$ for 30 days in a flow-through 500-L seawater tank for each group; the VHSV-challenged group was kept in a tank of the same volume at $16 \pm 1^\circ\text{C}$. Five fish in the control group was injected with PBS and kept at $16 \pm 1^\circ\text{C}$ or $20 \pm 1^\circ\text{C}$. Cumulative mortalities of five groups challenged with *E. tarda* NUF806 cells on the 0.5% and 3% NaCl agar, *S. iniae*, *S. parauberis*, VHSV and two control groups at 20°C and 16°C were 46.7, 50.0, 60.0, 80.0, 60.0, 0 and 0%, respectively. In each group, five fish were randomly-selected from the survival at 30 days after the challenge, and then these sera were pooled and stored at -80°C until used. Production of specific antibody against each pathogen was confirmed by slide agglutination tests with formalin-killed cells of each bacterium or by an indirect fluorescent antibody test against VHSV

JF00Ehi1.

Results of the immuno-dot-blot test of each serum sample are shown in Fig. 2. The antibody reaction against the recombinant protein was detected in sera from fish injected with *E. tarda* grown on the 3% NaCl agar but not in the other sera. Yasunobu *et al.*⁹⁾ also detected the fimbrial major subunit protein in *E. tarda* grown in a culture containing 3% NaCl but not in cultures containing $<1.5\%$ NaCl. In this study, the fimbrial major protein was detected in *E. tarda* NUF806 cultured on the 3% NaCl agar by the immunoblotting analysis but not in the cells on the 0.5% NaCl agar (Fig. 3). In addition, the fimbrial protein was not observed in the bacterium grown on the 0.5% NaCl agar after the cultivation on the 3% NaCl agar. Furthermore, the protein was not shown in the bacterium passaged on the 0.5% NaCl agar. These results suggested that high concentration of NaCl was one of induction factors for generation of fimbriae in *E. tarda* and that the expressed fimbrial protein was recognized as an antigen by the immune system of Japanese flounder. In the challenge test with *E. tarda* cultivated on the 3% NaCl agar, the living bacterium was isolated from all sampled flounder having the specific antibody against the fimbrial protein. And the specific antibody was not observed in flounder challenged with *E. tarda* grown on the 0.5% NaCl agar. The stability of the expressed fimbrial protein in the fish body is still unknown. The further study is still needed for clarification of the fimbrial function in the virulence of *E. tarda*.

Serum was periodically collected from 10 Japanese flounder randomly sampled at a fish farm in Oita Prefecture, Japan, from August to December 2008.

Fig. 2. Antibody reaction of recombinant fimbrial protein in serum from Japanese flounder determined by the immuno-dot-blot test. Serum samples were obtained from fish injected with *E. tarda* grown on 0.5% NaCl agar (A); *E. tarda* grown on 3% NaCl agar (B); *S. iniae* (C); *S. parauberis* (D); VHSV (E); or PBS at 20°C (F).

Fig. 3. Immunoblotting analysis of *E. tarda* NUF806 with a rabbit antiserum against fimbrial protein. The bacterium on the 0.5% NaCl agar (A) was cultured on the 3% NaCl agar (B). After the cultivation with the 3% NaCl agar, the bacterium was passaged twice on the 0.5% NaCl agar (C and D). * Positive reaction against the fimbrial protein.

Fig. 4. Cumulative mortality of cultured Japanese flounder and water temperature in a farm. Arrows, fish and the serum sampling days.

Cumulative mortality of the fish and water temperature in the farm are shown in Fig. 4. During the sampling period, bacterial diseases due to *E. tarda* and *S. parauberis* had occurred in the farm. The causative agent was isolated from the brain or kidney with *Salmonella-Shigella* agar (Nissui) or Trypto-soya agar (Nissui) containing 2% NaCl at 25°C and identified by the slide agglutination test with rabbit antiserum prepared against each bacterium. Results of the immunodot-blot test for each fish serum sample are shown in Table 1. The fish having the antibody against the fimbrial protein was observed on each sampling day. The number of the positive fish increased with the occurrence of death starting in the late August. This sug-

gests that *E. tarda* expressing the fimbria infected Japanese flounder in the farm. In September, October and November, the fish without *E. tarda* was found in some fish showing the positive reaction. Although these fish have experienced the infection of *E. tarda*, the infected *E. tarda* would be killed by the host defense mechanism. The fimbrial protein may be immunogen against edwardsiellosis. On the other hand, *E. tarda* carrier was observed in the fish showing the negative reaction in the antibody test on each sampling day except November 14. Matsuyama *et al.* reported that some immune-related genes, i.e. MHC class II and immunoglobulin, of Japanese flounder have been down regulated for a few days after infection of *E. tarda*²⁰⁾. Such carrier showing the negative reaction might not have been producing the antibody against fimbria in early phase of the infection.

The findings of the present study suggested that *E. tarda* with the fimbria was important for epidemics of edwardsiellosis at flounder farms. Some novel genes of putative adhesins have recently been identified in *E. tarda* by full-genome sequencing²¹⁾. The fimbria may serve as one of the adhesins for the infection to Japanese flounder. To understand the occurrence of disease and the infection process of *E. tarda*, further studies are needed regarding the detailed expression and function of fimbria.

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Table 1. Antibody reaction of Japanese flounder in a farm against *E. tarda* fimbrial recombinant protein

Fish No.	Antibody reaction of (Bacteria isolation from) the farmed fish on						
	Aug. 28	Sept. 9	Sept. 22	Oct. 9	Oct. 22	Nov. 14	Dec. 11
1	+*(Et) [†]	+	+(Et, Sp)	+(Et)	+(Et)	+(Et)	+(Et)
2	+(Et)	+	+(Sp)	+	+(Et)	+(Et)	+(Et)
3	-(Et)	+	+	+	+(Et)	+(Sp)	+
4	-(Et)	-(Et, Sp)	+	-(Et)	+(Sp)	+(Sp)	+
5	-(Et)	-(Sp)	-(Et)	-(Et)	+(Sp)	-	+
6	-(Et)	-(Sp)	-(Et)	-(Et)	+	-	+
7	-(Sp)	-(Sp)	-(Et)	-(Et)	+	-	-(Et)
8	-(Sp)	-	-(Sp)	-(Et)	-(Et)	-	-(Et)
9	-	-	-(Sp)	-	-	-	-(Et)
10	-	-	-(Sp)	-	-	-	-

* = antibody-positive reaction; - = negative reaction.

[†] Et = *Edwardsiella tarda*; Sp = *Streptococcus parauberis*.

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