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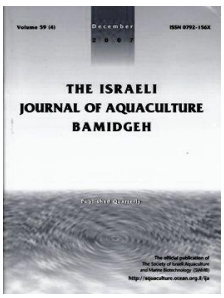

Visualization of *Sparus macrocephalus* Infection by GFP-Labeled *Edwardsiella tarda*

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Abstract

Edwardsiella tarda is a fish pathogen that causes systemic infections in many marine and freshwater fish. The invasion pathway of *E. tarda* was studied *in vivo* in *Sparus macrocephalus* using a virulent strain of *E. tarda*, transformed with a plasmid encoding green fluorescent protein and sharing similar virulence characteristics as the parent strain. Fish were divided into six groups: (A) bacteria bath-challenged, unwounded fish, (B) bacteria bath-challenged fish, skin mucus layer partially removed by paper towel, (C) bacteria bath-challenged fish, skin artificially wounded by scalpel, (D) bacteria intramuscularly-challenged fish, (E) normal fish, and (F) phosphate-buffered saline intramuscularly-treated fish. The number of bacteria from blood, gills, kidney, muscle, spleen, liver, and intestine were determined 2, 4, 6, 12, 24, and 48 h post-challenge. High numbers of bacteria were observed in the gills and intestine, followed by the liver, kidney, spleen, and muscle of infected fish. The number of bacteria was significantly higher in groups B and C than in group A. The skin, intestine, and gills were likely the main routes of entry for *E. tarda*.

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Introduction

Edwardsiella tarda is an intracellular, rod-shaped gram-negative, non-capsulated, motile, facultative anaerobic bacterium that was first isolated from a pond-cultured eel by Hoshina (1962). *Edwardsiella tarda* is widely distributed in aquatic environments (Wyatt et al., 1979; Pitlik et al., 1987) and is infectious to a variety of animals including humans (Clarridge et al., 1980; Janda and Abbott, 1993), fish (Han et al., 2006; Padrós et al., 2006), amphibians (Sharma et al., 1974), reptiles (Sechter et al., 1983; Sugita and Deguchi, 1983), and birds (Winsor et al., 1981; Cook and Tappe, 1985). This organism's versatility with respect to the broad range of hosts highlights the importance of developing strategies to understand the interactions between *E. tarda* and its hosts.

The use of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* as an endogenous fluorescent tag provides a means of rendering bacteria visible and tracing their activity in living host cells (Prasher et al., 1992; Chalfie et al., 1994; Ling et al., 2000). GFP has the property of fluorescing when excited by ultraviolet (UV) light (Chalfie et al., 1994). The GFP fluoresce is independent of cofactors, substrates, or any additional gene product, is sensitive, stable, specific, non-toxic, and does not interfere with cell growth and function (Ling et al., 2001). With such a tool, invasion pathways of *E. tarda* in blue gourami were analyzed using small doses of bacteria to mimic the process of naturally acquired *E. tarda* infections (Ling et al., 2001).

The objective of this study was to use GFP as a biomarker to illustrate the infection kinetics and tissue localization of *E. tarda* in *Sparus macrocephalus* after bath and intramuscular challenge.

Materials and Methods

Bacterial strains and media. The SM strain *of E. tarda* was isolated from cultured *S. macrocephalus* that died from a natural outbreak of epidemic septicemia in Wenzhou, China. The bacteria was cultured in tryptic soy broth (TSB, Oxoid) or on tryptic soy agar (TSA) at 27°C for 24 h. Stock cultures were maintained at -20°C in a suspension of TSB containing 30% (v/v) glycerol. When selecting the GFP-marked bacteria, the antibiotic carbencillin (sangon) was added at a final concentration of 50 μg/ml.

Transformation of E. tarda strain SM with pFPV25.1. Plasmid pFPV25.1 was transformed into *E. tarda* strain SM using a standard electro-transformation protocol (Valdivia et al., 1996). Plasmid pFPV25.1 has an carbencillin resistance marker and transformed *E. tarda* were plated on TSA containing carbencillin (50 μg/ml). Colonies that were resistant to carbencillin and fluoresced bright green under UV light (Liuyi) were selected, examined under a fluorescence microscope (Zeiss), and identified by PCR with oligonucleotides GFP-forward (5'-GTCATGCGTGATGCAATG-3') and GFP-reverse (5'-CATTTATTTGTATAGTTC-3'). PCR (BIO-RAD) amplification steps consisted of one 4-min cycle at 95°C, 30 cycles at 94°C for 45 s, 56°C for 50 s, and 72°C for 90 s, and a final extension step at 72°C for 10 min. The PCR product was visualized on 1.0% agarose gel stained with ethidium bromide. The transformed *E. tarda* strain SM was designated *E. tarda* SM-GFP.

Plasmid stability. To check the ability of *E. tarda* SM-GFP to retain the plasmid, a single colony of the bacteria was inoculated into a test tube containing 5 ml carbencillin-free TSB. This bacterial culture was continuously subcultured by reinoculating into a fresh 5-ml TSB media daily for 7 days. The culture was sampled daily to quantify the number of bacteria by plate counting. Plasmid stability was checked by examining the ability of the bacterial colonies to fluoresce under an UV lightbox (Chu and Lu, 2008).

Comparison of E. tarda strains SM and SM-GFP. Hemolytic activity was determined by measuring the zone of hemolysis around colonies on blood agar plates as described by Imziln et al. (1996) with a little modification. The strains were grown on blood agar plates (TSA supplemented with 5% goat blood) at 27°C for 48 h, and the diameters of the hemolytic zones were recorded.

To detect serum survival, bacterial suspensions in sterile saline (0.5 ml each; 10^8 viable cells/ml) were added to fresh normal *S. macrocephalus* serum (0.5 ml) or rabbit anti-*E. tarda* SM serum (0.5 ml). They were mixed, incubated in tubes with occasional

shaking at 28°C for 2 h, and plated onto *Salmonella shigella* agar (SSA, Luqiao). The presence of *E. tarda* was confirmed by quantifying colonies with black pigmentation.

To determine 50% lethal doses (LD_{50}), five groups of ten *S. macrocephalus* (210±10 g) were obtained from an aquaculture farm in Wenzhou, Zhejiang Province, China, kept in 2000-l tanks supplied with sea water, and fed a commercial pelleted diet twice a day. The water temperature was kept at 24±2°C. The experimental fish were acclimatized in the experimental tanks for at least two weeks. Each was injected intramuscularly with a 0.1-ml dose of washed bacterial cells adjusted to the required concentration and monitored for 7 days. For mortality studies and all fish at the end of the experiment, muscle tissue at the injection site was streaked on SSA and *E. tarda* infection was confirmed by quantifying colonies with black pigmentation. LD_{50} values were calculated by the method of Reed and Muench (1938).

Fish. Sparus macrocephalus (210±10 g) were obtained from a fish farm in Zhejiang Province, China. They were kept in a 2000-l recirculating aquaria with sea water at a constant temperature of 24±2°C and fed commercial feed every day. The fish were maintained in the tanks for 14 days to ensure they had no diseases. The fish were divided into six treatment groups as follows: (A) bacteria bath-challenged, unwounded fish, (B) bacteria bath-challenged fish, skin mucus layer partially removed by paper towel, (C) bacteria bath-challenged fish, skin artificially wounded by scalpel, (D) bacteria intramuscularly-challenged fish, (E) normal fish, and (F) phosphate-buffered saline intramuscularly-treated fish.

Study of infection kinetics. The intramuscular route of administration was used to study the infection kinetics of *E. tarda in vivo*. Fish were infected with GFP-marked *E. tarda* by bath or intramuscular administration. *Edwardsiella tarda* SM-GFP was cultured in TSB at 28°C for 24 h, shaking at the speed of 200 rpm, and bacteria were counted. Fish in groups A, B, and C were exposed to bacteria at a final concentration of 1.0×10^7 cfu/ml for 60 min, then stocked in 2000-l clean recirculating sea water. Fish of group D were injected intramuscularly with 0.1-ml doses of washed bacterial cells adjusted to 1.0×10^7 cfu/ml. Fish of groups E and F were treated with PBS, by bath and intramuscularly, respectively. Three fish in each group were sampled 2, 4, 6, 12, 24, and 48 h after bath or intramuscular challenge. The fish were anesthetized with tricaine methane sulfonate (MS-222, Sigma) at a concentration of 400-500 mg/l and blood, gill, intestine, body muscle, kidney, and liver were aseptically removed. Blood was collected aseptically from the caudal vein. A piece of body muscle (0.5 × 0.5 cm) was dissected from opposite the site of the wound and weighed. The gill was washed 3 times with PBS to remove surface bacteria. Samples were placed into separate sterile sample bags, diluted with PBS at a final concentration of 0.1 g/ml, and homogenized. The homogenates were serially diluted, plated onto TSA containing carbencillin, and incubated at 28°C for 24 h. Bacteria were counted with an UV lightbox.

Statistical analysis. Statistical significance was determined by ANOVA analysis. Differences were considered significant at $p<0.01$ or $p<0.05$.

Results

Transformation of E. tarda with pFPV25.1. The pFPV25.1 plasmid successfully transformed *E. tarda* strain SM into SM-GFP which fluoresced an intense green when illuminated with UV light. The GFP gene was found by PCR (Fig. 1). The *E. tarda* SM-GFP fluoresced when examined under a fluorescence microscope (Fig. 2). Plasmid in the bacteria, tested *in vitro* by subculturing in carbencillin-free TSB daily for 7 days, was maintained at 100% (n = 3) in the strain at the end of culture.

Characteristics of transformed E. tarda SM-GFP. The SM and SM-GFP strains had similar fish LD_{50} values, responses to normal serum and anti-serum, and hemolysin production (Table 1).

Infection kinetics in Sparus macrocephalus. Bacteria were detected in all infected groups but not from the uninfected control groups E and F (Fig. 3). Generally, the number of bacteria was greatest in the intestine and gill on day 2, followed by the liver, kidney, spleen, and muscle.

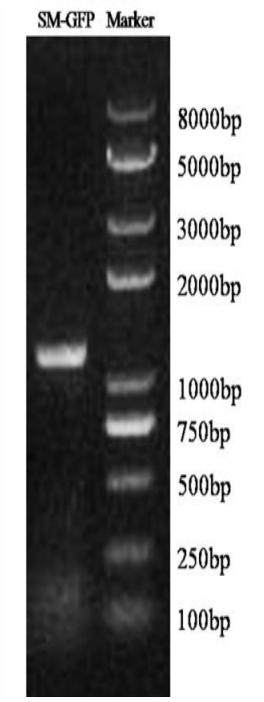

Fig. 1. Presence of the GFP gene in *Edwardsiella tarda* SM-GFP; marker = DL2000 Plus II.

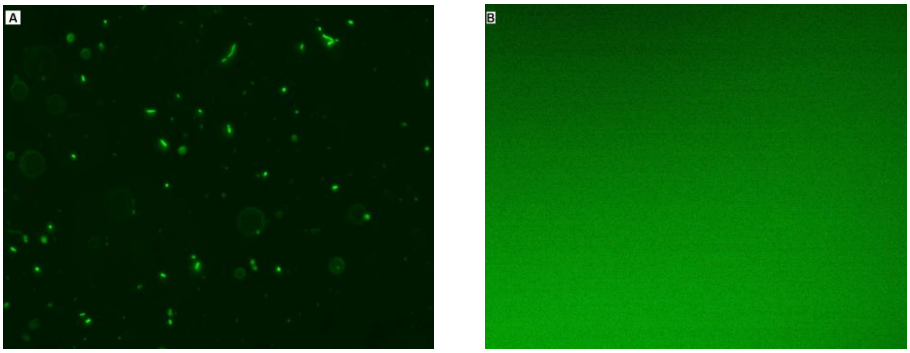

Fig. 2. (A) *Edwardsiella tarda* SM-GFP and (B) *E. tarda* SM illuminated with fluorescence microscope.

Table 1. Characteristics of the GFP-tagged *Edwardsiella tarda* strain and the parent strain.

Strain	*Hemolysin*[1]	*Response to normal serum and anti-serum*[2]	*LD_{50} (intramuscular injection)*[3]
E. tarda SM	+	Resistant	8.06×10^4
E. tarda SM-GFP	+	Resistant	8.31×10^4

[1] Measured by the diameter of clear zones after 24 h incubation on TSA containing 5% goat blood. Data obtained from three independent experiments.
[2] Fresh normal serum and anti-*E. tarda* SM serum were used. Resistant = less than 80% reduction in viable counts due to serum; sensitive = greater than 3 logarithms of reduction in viable counts due to serum.
[3] Calculated by the method of Reed and Muench (1938). Sample size was 10 fish per tank.

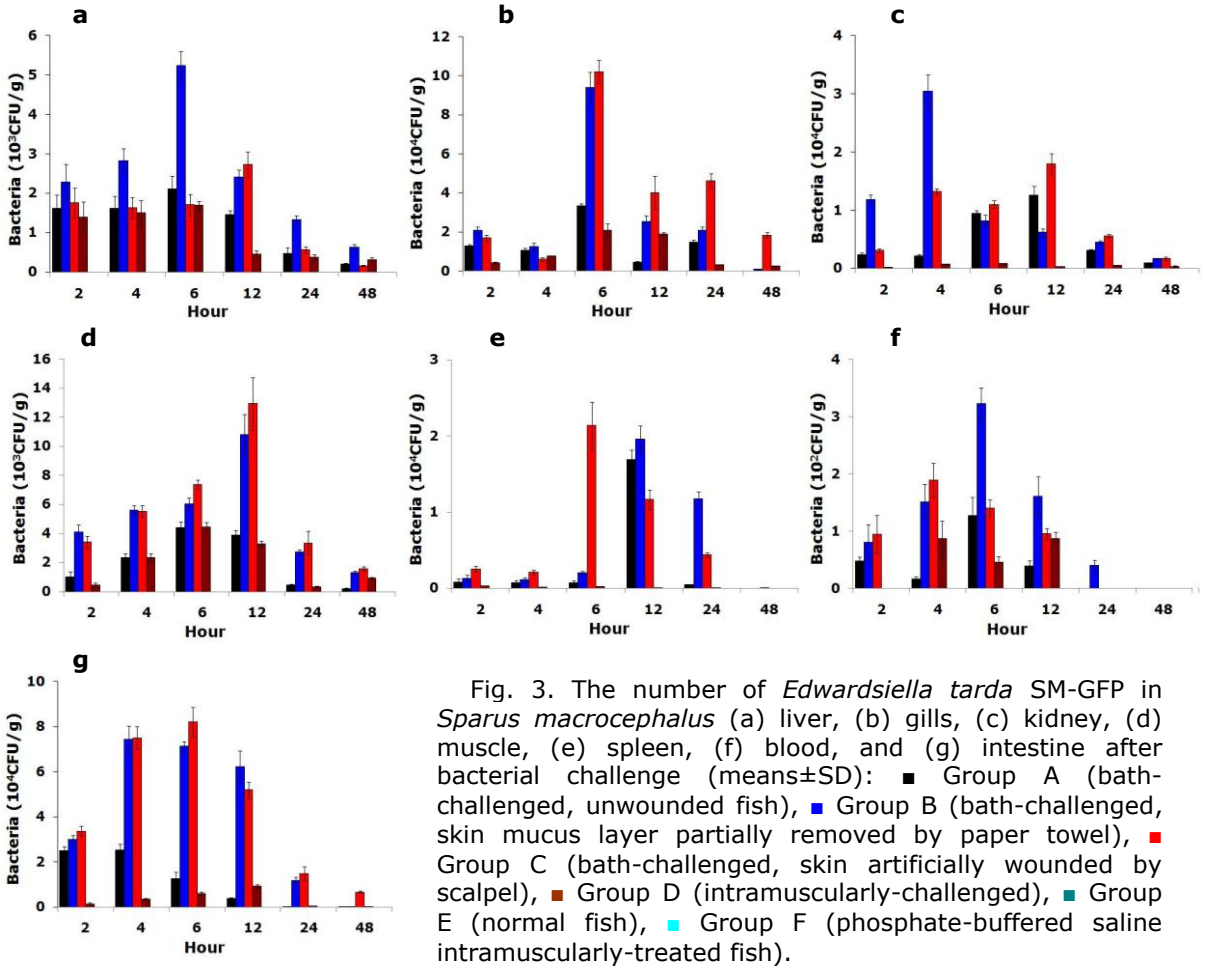

Fig. 3. The number of *Edwardsiella tarda* SM-GFP in *Sparus macrocephalus* (a) liver, (b) gills, (c) kidney, (d) muscle, (e) spleen, (f) blood, and (g) intestine after bacterial challenge (means±SD): ■ Group A (bath-challenged, unwounded fish), ■ Group B (bath-challenged, skin mucus layer partially removed by paper towel), ■ Group C (bath-challenged, skin artificially wounded by scalpel), ■ Group D (intramuscularly-challenged), ■ Group E (normal fish), ■ Group F (phosphate-buffered saline intramuscularly-treated fish).

Discussion

Information on the portals of entry for fish pathogens comes from observations of the surface tissues of fish using microscopic, microbiological culture, or radioactive tracer techniques following exposure of the host to the pathogen (Amaro et al., 1995; Svendsen et al., 1999). Conventional infection kinetics studies quantify bacteria populations in infected organs by culturing them on antibiotic-containing plates, an indirect method that may overestimate the number of bacteria (Chu and Lu, 2008). This point is important when bacteria samples come from the gastrointestinal tract where a wide variety of bacteria are present. Identification of the bacteria is tedious and may be inaccurate. Radioiodinated bacteria can be detected quantitatively, but the disadvantage to this method is that it does not discriminate between live and dead bacteria (Chu and Lu, 2008). In the present study, the chosen bacterial strain was transformed by a GFP-carrying plasmid. The transformed strain and the parent strain were characterized by virulence factors and found to be similar. Thus, the transformed strain could be used as a representative of the virulent strain. Immersion and injection infection models simulate natural infection conditions. Our results indicate that the GFP label is an ideal tool for studying the invasion route of fish pathogens in fish.

It has been postulated that skin is the portal of entry for fish pathogens such as *Vibrio anguillarum* (Kanno et al., 1989), *Aeromonas salmonicida* (McCarthy, 1983), and *A. hydrophila* (Chu and Lu, 2008). Likewise, *E. tarda* adhere to the fish surface during immersion infection (Ling et al., 2001). Necessarily, fish skin has mechanisms that defend against invasion of pathogens carried by water, which is a perfect medium for many bacteria and parasitic microbes. In addition to the fish surface barrier, innate immune factors play important roles in the defense systems of fish. Lectins and other carbohydrate-binding proteins in the skin mucus might be part of the external barrier against pathogens. Immunoglobulins, T cell markers, cytokines, and complement factors are also present in mucus (Chu and Lu, 2008). Virulent *E. tarda* may be able to resist the host immune defense system, adhering and surviving in the mucus and proliferating inside the fish. After adhering and proliferating on the body surface of infected fish, virulent *E. tarda* can invade the muscle tissue underneath, causing obvious body lesions and hemorrhages (Ling et al., 2001). In this study, groups B and C, which had damaged skin, also had the highest amounts of bacteria in tissues/organs, indicating that fish skin was the primary route of bacteria entry. Virulent *E. tarda* may escape the immune defense system of the host, adhere and proliferate on the body surface of infected fish, and then invade the muscle tissue.

Fish gills may also be a portal of entry for *E. tarda*. Gills are structured so that a single layer of fragile cells separate the vascular system of the fish from the external environment. Gills are highly vascularized, with a large number of blood capillaries for respiratory functions, which make them good entry sites for disseminating bacteria to the rest of the fish (Ling et al., 2001). In our study, the numbers of bacteria in gills in all groups were high, supporting the finding that this organ is a key portal of entry for fish pathogens (Ling et al., 2001). Gills are a site of entry for other fish pathogens such as *V. anguillarum* and *A. hydrophila* (Laurencin and Germon, 1987; Chu and Lu, 2008).

The gastrointestinal tract is an entry site in fish for bacteria such as *V. anguillarum* and *A. hydrophila* (Olsson et al., 1996; Chu and Lu, 2008) and may be the primary portal of entry for *Edwardsiella ictaluri* and *E. tarda* (Baldwin et al., 1993; Ling et al., 2001). In our study, the numbers of bacteria in the intestines of all groups were high, suggesting that the gastrointestinal tract is a key portal of entry for *E. tarda*, similar to other food-borne enterobacteria that survive through the stomach before reaching the intestine.

The blood and kidney are the most important tissues for clearing fish pathogens from the bloodstream in rainbow trout and crucian carp (Ferguson et al., 1982; Chu and Lu, 2008). In this study, the numbers of bacteria in the blood of infected groups were low while the numbers of bacteria in the kidney, liver, muscle, spleen, and intestine were high throughout the observed period, suggesting that the *E. tarda* load in these organs is condition-dependent. There was rapid clearance of bacteria from the fish in all infected

groups after 24 h, suggesting that although bacteria entered the body, it did not result in sustained infection as observed by Ling et al. (2001).

In all, *E. tarda* were observed at the three principal entry points of the fish infection model - skin, gills, and intestine. These sites are in constant contact with the external medium, making them vulnerable and accessible to pathogen attack. The use of fluorescent proteins is an effective, direct, and visual means of detecting *E. tarda* in the presence of other bacteria. The combination of immersion challenge and GFP labeling is an ideal tool for studying intimate host-pathogen interactions in fish. Further, this is an ideal experimental design for evaluating vaccines and finding the most effective route of administration by checking the entry, targeted organs, and persistence of a bacterium.

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