Evaluation of an Experimental Immersion Infection Method with *Tenacibaculum maritimum* in Japanese Flounder *Paralichthys olivaceus*

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**Abstract:** Necessary condition to perform experimental infection with *Tenacibaculum maritimum* was investigated. A shaking culture of the bacterium for 48 h was used to infect Japanese flounder *Paralichthys olivaceus*. First, three infection methods, intraperitoneal (IP) injection and two immersion methods were compared. IP injected flounder with *T.* *maritimum* at doses of $10^{4.82}$ or $10^{4.92}$ CFU/fish did not exhibit high mortality or typical symptoms. “Immersion and dilution method”, where immersion-infected fish at doses $10^{5.81}$ or $10^{5.91}$ CFU/ml were successively reared in the same tank supplying fresh rearing water, resulted in higher mortality than “immersion and transfer method”, where immersion-infected fish were transferred to another rearing tank. Using immersion and dilution method, stable mortality occurred between 17°C and 26°C but not below 17°C or over 26°C. Too much diluted bacterial culture with sea water did not yield high mortality even when the immersion water contained enough bacterial concentration. This suggests extracellular substance contains virulence factor. *T. maritimum* was detected on the body surface of infected fish but not in the gills or kidney. In conclusion, the immersion and dilution method is an adequate infection method for this disease. In order to achieve satisfactory infection, the precise management of water temperature and the dilution rate of bacterial culture are required.

**Key words:** *Paralichthys olivaceus*; *Tenacibaculum maritimum*; Experimental infection; Immersion infection

*Tenacibaculum maritimum* (formerly *Flexibacter maritimus*), a Gram-negative, filamentous and gliding bacterium, has been described as the etiological agent of the infectious disease tenacibaculosis occurring worldwide in marine fishes (McVicar and White 1979; Kusuda and Kimura 1982; Campbell and Buswell 1982; Wakabayashi et al. 1984; Baxa et al. 1986; Kent et al. 1988; Devesa et al. 1989; Bernardet et al. 1990, 1994; Alsina and Blanch 1993; Chen et al. 1995; Handlinger et al. 1997; Ostland et al. 1999; Cepeda and Santos 2002). This disease is associated with characteristic gross lesions on the body surface such as ulcers, eroded mouth, congestion, frayed fins and tail rot. Necrosis in the gills and eyes are occasionally observed (McVicar and White 1979; Campbell and Buswell 1982; Wakabayashi et al. 1984; Handlinger et al. 1997; Ostland et al. 1999; Cepeda and Santos 2002). However, these reported symptoms are representative ones that can be seen in heavily diseased individuals in the infected fish groups. Most of dying fish in mass mortalities exhibit very slight symptom on the fins and skins especially in small fry fish. Sodium nifrustyrenate is the only chemotherapeutic drug that can be employed against tenacibaculosis in Japan. Furthermore,
this drug was prohibited except for limited use for flatfish fry smaller than 50 g body weight. Therefore, an alternative method to control this disease is urgently needed.

To develop control measures, such as chemoprophylaxis, vaccines and immunostimulants, a reliable experimental infection method has to be established. To date, various infection methods to induce the disease experimentally have been evaluated to study the pathogenesis by this bacterium (Baxa et al. 1987; Handlinger et al. 1997; Avendaño-Herrera et al. 2006a, 2006b; Nishioka et al. 2009). A recently report by Nishioka et al. (2009) showed that high mortality was obtained by immersion method with relatively higher infection dose of *T. maritimum* in Japanese flounder.

In the present study, attempts were made to establish a reliable immersion infection method for tenacibaculosis of Japanese flounder *Paralichthys olivaceus* by close examination on various infection conditions.

**Materials and Methods**

**Bacteria**

Two strains of *T. maritimum* were used in this study. Strains 050603 and 46501 were isolated from external surface lesions of Japanese flounder in 2005 in Kochi Prefecture and in 1995 in Oita Prefecture, respectively. Bacteria were pre-cultured in 20 ml modified ZoBell 2216E (m-ZoBell; 0.5% w/v peptone, 0.1% w/v yeast extract, 80% w/v seawater, pH 7.2) broth at 25°C for 24 h, then 150 μl was inoculated into fresh 150 ml m-ZoBell broth in 200 ml flasks, which were then incubated at 25°C for 20-50 h while being shaken at 100 rpm.

**Experimental fish**

Several Japanese flounder (mean weight 4.7 – 45.8 g depending on the experiments) were obtained from Kagawa Prefectural Fisheries Experimental Station (Kagawa Prefecture, Japan). Fish were collected from production lots that had no record of any previous occurrence of tenacibaculosis. Fish were reared in 800 l FRP tanks with continuously flowing seawater at a temperature range from 15 to 29°C according to environmental seasonal change. The fish were fed a satiation amount of commercial dry pellets (Nissin, Japan) throughout the experimental periods, corresponding to about 3 to 8% of fish body weight depending on to fish size.

**Intraperitoneal (IP) injection**

Fifty fish in one group were injected IP with 0.1 ml of the suspensions of strain 050603 in PBS at doses of $10^{4.32}$ or $10^{4.82}$ CFU/fish. After injection, the fish were transferred into 200 l FRP tanks with continuous seawater supply.

**Immersion infection**

Two concentrations of infecting bacterial suspensions, about 10 times differently diluted bacterial cultures, were prepared in seawater by adding 150, 75 or 15 ml of bacterial broth culture to 20 l sea water or adding 400 or 40 ml of bacterial broth culture to 50 l seawater. Immersion infection was performed by two methods; “immersion and dilution” method and “immersion and transfer” method. In the immersion and dilution method, fish were infected in a bacterial suspension for 30 min then reared in the same tank supplying fresh seawater for 15 days. In the immersion and transfer method, fish were infected in a bacterial suspension for 30 min then transferred to another tank with fresh seawater supply and reared for 15 days. Control fish were treated by immersion in *T. maritimum*-free seawater and reared in the same tank. *T. maritimum* strain 050603 was used in all the experiments, and strain 46501 was used only in experiments to compare the two immersion infection methods. Table 1 shows the summary of the eight experiments using the immersion and dilution method. Bacterial suspensions in seawater were prepared with the following dilution rates: bacterial broth culture/seawater was 150 ml/20 l where fish number in one group was 30 or less, or 400 ml/50 l where fish number in one group was more than 30.

Effect of temperature was evaluated by above 8 experimental infections and 41 additional tests.
Detection of *T. maritimum* from infected fish

Ten fish (mean body weight 32.6 ± 3.7 g) were used for infected fish by immersion and dilution method. Another 10 fish were used as control. Fish from infection and control groups one day after infection were killed by dipping in an excess concentration of anesthetic. Tissue samples from the skin, gills and kidney were taken by using platinum loop then inoculated by streaking on m-ZoBell agar plates. The tissues were also imprinted onto glass slides, stained with methylene blue and examined under a microscope to observe bacterial cells. The specific detection of *T. maritimum* was performed on the tissue imprints by fluorescent antibody technique (FAT) using an anti-050603 rabbit serum. The rabbit antiserum, prepared in our laboratory, was used as the primary antibody at a dilution of 1:250, and a commercial goat anti-rabbit immunoglobulin antibody conjugated with tetramethyl rhodamine (Molecular Probes) was used as the secondary antibody at a dilution of 1:200.

Statistics

Statistical analysis were performed by *F*-test for the samples of over 30 individual/group and the Mann-Whitney *U*-test for samples of less than 30 individuals/group, using the extended statistical table of Rohlf and Sokal (1981). Significance was accepted for *P* < 0.05 in *F*-test and *P* < 0.05 in *U*-test.

Results

Bacterial growth

After about 24 h of incubation, shaken broth cultures formed slime-like aggregates suspended in the medium and attached to the walls of the culture flasks of strains 46501 and 050603. After bacterial aggregates were resuspended for 5 sec each time manually, the bacterial suspension was subjected to a bacterial count on agar plates and optical density determination for both strains. This also reduced the effect by aggregates and slime in the broth culture. Figure 1 shows the growth curve obtained from the suspension of the shaken culture of strain 050603.

The viable bacterial count increased quickly for 36 h and then slowly until 54 h.

Experimental infection

To select on adequate growth time, a preliminary virulence experiment was performed using the immersion and dilution method using 20 h and 50 h cultures of strain 050603. As both cultures showed similar virulence (Fig. 2), 48 h cultured bacteria were used in the following infection experiments.

Mortality profiles for the three infection methods are shown in Fig. 3. IP injection at doses of 10^4.92 and 10^4.82 CFU/fish showed obviously lower mortality than immersion infection method with strain 050603 at doses of 10^5.91 and 10^5.81 CFU/ml (Figs. 3A, 3B). *T. maritimum* was isolated from various sites on the skin of immersion infected fish. In IP injected fish, the bacterial was isolated from the ascitic fluid and injection site of the skin but not from other
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Strain 050603 resulted in similar mortality for both immersion methods, however strain 46501 showed higher mortality rate by the immersion and dilution method than the immersion and transfer method (Figs. 3C, 3D). A high mortality was achieved with bacterial doses of 10^{5.54} to 10^{6.85} CFU/ml. Figure 4 shows the results of the immersion and dilution infection experiments, where the two dilutions of bacterial broth cultures differed by one order of magnitude. The higher dilution rate of the broth culture with seawater (15 ml/20 l) lowered mortality even though bacterial doses were higher than 10^6 CFU/ml. Table 1 shows that infection doses upper than 10^{5.5} CFU/ml resulted high mortality (greater or equal 79%) regardless of fish size.

Fig. 3. Mortality profile of Japanese flounder by different infection methods using T. maritimum strains 050603 (A and B) and 46501 (C and D). , immersion and dilution; , immersion and transfer; , intraperitoneal injection (only for strain 050603); , control. Significant difference: *, P<0.05 by F test; #, P<0.05 by U test.

Fig. 4. Mortality of Japanese flounder following infection by immersion and dilution method with different doses of T. maritimum strain 050603 and different dilution rates of broth culture. Experiment 1: , 10^{6.70} CFU/ml (dilution rate, 150 ml culture/20 l sea water); , 10^{6.40} CFU/ml (dilution rate, 75 ml culture/20 l sea water); , control. Experiment 2: , 10^{6.70} CFU/ml (dilution rate, 150 ml culture/20 l sea water); , 10^{6.00} CFU/ml (dilution rate, 15 ml culture/20 l sea water); , control. Experiment 3: , 10^{5.56} CFU/ml (dilution rate 400 ml culture /50 l seawater); , 10^{5.00} CFU/ml (dilution rate 40 ml culture/50 l sea water); , control. Significant difference: # and ##, P<0.05 and P<0.01 respected by U test; ***, P<0.01 by F test.

Fig. 5. Mortality of Japanese flounder following infection by immersion and dilution method with T. maritimum strain 050603 at different water temperatures. Bars without standard error indicate results from single infection experiments.
Figure 5 summarizes the result of 49 experiments assessing the effect of water temperature on experimental infection with strain 050603. Relatively stable mortality was obtained at a range between 17°C and 26°C, but variable and lower mortality was observed below 17°C and above 26°C.

**Clinical signs**

Fish showed typical symptoms of tenacibaculosis such as mouth congestion, eroded body surface and fin rot, which were similar to those observed in naturally infected fish. The gills, kidney and spleen did not show any symptoms. IP injected fish did not show any clinical signs on their body surface, but showed the accumulation of ascitic fluid in the peritoneal cavity and liver congestion (data not shown).

**Detection of T. maritimum in tissue samples**

Methylene blue stain showed numerous bacteria on the body surface (Fig. 6A). However, only a few bacteria were observed in the gills, and none in the kidney (Figs. 6B, 6C). Likewise, *T. maritimum* was detected only on the body surface by FAT (Fig. 6D) but not in the gills or kidney (Figs. 6E, 6F) or on any site in the control fish.

**Discussion**

Immersion infection has been widely used in the experimental infection of fish because its infection process that placing fish in a suspension of pathogenic microbes is probably closer to naturally occurring infection than that of injection method. However, satisfactory results in mortality and symptom reproducibility in the infected fish are not always achieved. The results may differ depending on pathogens, fish species, environmental conditions and personal skills. Experimental conditions such as infection dose, water temperature, immersion time, use of anesthesia *etc.* are usually selected according to a compromise between convenience and reality. For example, Avendaño-Herrera et al. (2006a) reported that 18 h of immersion was required for successful infection of turbot *Scophthalmus maximus* with *T. maritimum*, while a shorter immersion could not achieve adequate infection. However, a long immersion is not convenient for practical infection experiments. Furthermore, physiological condition for experimental fish may deteriorate by being kept in the limited volume of immersion water and tank space. The present study attempted
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to infect Japanese flounder experimentally with *T. maritimum* by immersing fish for a limited time 30 min and to determine adequate infection conditions. Moderately high mortality was achieved in shorter immersion time than that reported by Avendaño-Herrera et al. (2006a). The difference may be related to the bacterial strain and culture method and/or to the fish species.

Recent reports have demonstrated that the fish pathogen *Flavobacterium psychrophilum* and *Photobacterium damselae* subsp. *piscicida* showed high virulence at logarithmic culture phase (Kondo et al. 2001; Nagano et al. 2009). *F. psychrophilum* is a fish pathogen phylogenetically related to the genus *Tenacibaculum*, which changes its virulence as well as cell forms during logarithmic and stationary culture phases (Kondo et al. 2003; Aoki et al. 2005). In the present study, the 20 h and 50 h cultures of *T. maritimum* produced similar infection rates. Therefore, it could be concluded that the culture time of *T. maritimum* does not affect virulence in 50 h culture time. Campbell and Buswell (1982) reported that *T. maritimum* grown in shaken broth culture produced mycelial-type aggregates on the bottom and sides of flask and occasionally formed aggregates in a ring form at the edge of broth area on the inner surface of flask. In present study, *T. maritimum* cultured for about 24 h also formed much amount of flocculent aggregates, which made it difficult to prepare a homogeneous bacterial suspension. Therefore, 48 h culture, which produced less amount of aggregates, was employed by using only the upper part of the broth culture after a 5 min aggregate sedimentation process. Attempts to resuspend aggregated bacterial cells by using detergents, sonication or crushing with glass beads were not employed because they

### Table 1. Summary of experimental conditions by immersion and dilution infection experiments with *Tenacibaculum maritimum* strain 050603

<table>
<thead>
<tr>
<th>Experiment and group</th>
<th>Fish size (g) (± SE)</th>
<th>Water temperature (°C) (± SE)</th>
<th>Dose (Log CFU ml⁻¹)</th>
<th>Dead fish/used fish</th>
<th>Mortality (15 day post infection, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>4.7 (± 1.3)</td>
<td>24.5 (± 0.5)</td>
<td>6.85</td>
<td>60/60</td>
<td>100</td>
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<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>0/60</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>4.8 (± 0.9)</td>
<td>21.5 (± 0.5)</td>
<td>5.54</td>
<td>60/60</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>0/60</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>7.5 (± 2.1)</td>
<td>22.5 (± 0.5)</td>
<td>6.30</td>
<td>40/40</td>
<td>100</td>
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<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>0/40</td>
<td>0</td>
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<tr>
<td>Experiment 4</td>
<td>13.2 (± 4.4)</td>
<td>23.5 (± 0.0)</td>
<td>5.79</td>
<td>38/40</td>
<td>93</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>0/40</td>
<td>0</td>
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<tr>
<td>Experiment 5</td>
<td>17.1 (± 5.4)</td>
<td>24.0 (± 0.5)</td>
<td>6.15</td>
<td>60/60</td>
<td>100</td>
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<td>Control</td>
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<td>0/60</td>
<td>0</td>
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<tr>
<td>Experiment 6</td>
<td>22.6 (± 7.0)</td>
<td>21.0 (± 0.0)</td>
<td>5.56</td>
<td>47/50</td>
<td>94</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>4/50</td>
<td>2</td>
</tr>
<tr>
<td>Infection 2</td>
<td></td>
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<td>Control</td>
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<td></td>
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<td>0/50</td>
<td>0</td>
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<tr>
<td>Experiment 7</td>
<td>29.2 (± 7.9)</td>
<td>18.0 (± 0.5)</td>
<td>6.45</td>
<td>25/25</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>0/25</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 8</td>
<td>45.8 (± 12.7)</td>
<td>23.0 (± 0.5)</td>
<td>6.51</td>
<td>25/32</td>
<td>79</td>
</tr>
<tr>
<td>Infection</td>
<td></td>
<td></td>
<td></td>
<td>0/30</td>
<td>0</td>
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may affect bacterial virulence or other pathogenic characteristics. Sedimentation did not affect these bacterial properties and this represented a better method to prepare the bacterial suspensions of *T. maritimum*.

Recently, Nishioka et al. (2009) reported that the satisfactory concentration of *T. maritimum* for experimental immersion infection was over $10^7$ CFU/ml and $10^6$ CFU/ml or less dose resulted low mortality. From the results of present infection experiments, the dose range for successful immersion infection by *T. maritimum* was estimated to be in the range from $10^{5.56}$ to $10^{6.65}$ CFU/ml. It should be noted that too much dilution of infecting culture broth failed to achieve adequate infection even if the bacterial dose was higher than $10^{5.56}$ CFU/ml. When the successful infection dose was included in 150 ml culture broth mixed with 20 l seawater (1:134.3 diluted) or 300 ml broth with 50 l seawater (1:167.7 diluted), infection was achieved. However, when same bacterial dose was included in 15 ml broth mixed with 20 l seawater or 30 ml broth with 50 l seawater (1:1667.7 diluted) high mortality was not achieved. A recent study reported that the extracellular protein of *T. maritimum* had a lethal effect to Atlantic salmon when IP injected (van Gelderen et al. 2009). If some extracellular virulence factor contributes also in immersion infection, this could explain the decreased mortality which was observed when bacterial broth is diluted 1,000 times or more.

In the experiment comparing two immersion infection methods, the immersion and dilution method was more successful in causing mortality. One of the reasons may be that after immersion time, fish were still being exposed to diluted bacterial suspension while fresh rearing water was being supplied. Another reason can be derived from following reports. Avendaño-Herrera et al. (2006a) reported that the bacteria attached to the body surface were detached when infected fish were handled roughly in seawater. Kawahara and Kusuda (1998) demonstrated that the cell surface of *T. maritimum* did not bear hydrophobic properties, therefore they suggested that the bacteria had a weak adhering ability. Our results and these reports indicate that the immersion and dilution method infection is recommended for the experimental infection of Japanese flounder with *T. maritimum*. Nishioka et al. (2009) reported that high mortality was achieved in Japanese flounder by immersion and transfer method with *T. maritimum*. This result was ascribable by high bacterial dose and long immersion time. In order to achieve high mortality with lower infection dose and low stress in treating experimental fish, immersion and dilution method in more suitable than immersion and transfer method.

It is reported that *T. maritimum* can grow at a temperature range between 15 to 34°C with the optimum growth temperature of 30°C (Wakabayashi et al. 1984). Present result that optimal temperature range was from 17 to 26°C demonstrates that the high virulence of this bacterium appears at a lower temperature than that reported for optimal growth.

In infected fish by immersion and dilution method, bacteria were detected only on the skin, while the gills and internal organs did not exhibit bacterial existence or any pathological sign. Conversely, when red sea bream *Pagrus major* and black sea bream *Acanthopagrus schlegeli* were experimentally infected by *T. maritimum* using smear or immersion infection, bacteria were detected in the skin, gills, liver and spleen (Kimura and Kusuda 1983; Baxa et al. 1987). In a natural infection case of tenacibaculosis in Atlantic salmon *Salmo salar* the bacteria were found to be adhering to the gills and damaging them (Handlinger et al. 1997). There have been many reports that gliding bacteria are frequently detected on the gills of affected fish. However, Kondo et al. (2002) demonstrated that *Flavobacterium psychrophilum* was not observed on the gills of ayu *Plecoglossus altivelis* after 24 h of immersion infection when the progress of pathogenesis had already started on the skin of the fish. Similarly to the report of Kondo et al. (2002), very few *T. maritimum* were detected on the gill of dead Japanese flounder in present experimental infection. The gills may have an antimicrobial function to eliminate bacterial invasion.
Dead fish by present immersion infection exhibited the erosion of body surface, congestion of mouth and operculum and fin rot, but did not show any symptoms in visceral organs. Miyazaki et al. (1975) also reported that yellow-tail *Seriola quinqueradiata* naturally infected by gliding bacteria did not show any pathological change in the spleen, liver and kidney. In the present study, IP injected fish showed accumulation of ascitic fluid in the peritoneal cavity without exhibiting any external signs. These results indicate that *T. maritimum* can not grow in inner organs but affects principally the outer tissue of fish.

In heavily infected and dead fish are occasionally accompanied by the successive proliferation of various bacteria usually occurred on gills and body surface and in internal organs as observed in red sea bream and Atlantic salmon (Kimura and Kusuda 1983; Handliger et al. 1997). However in the present study, very few other bacteria occurred in fish infected with *T. maritimum* using immersion method. Immersion and dilution method and adequate sampling of infected fish may enable to monitor the infection process of tenacibaculosis in Japanese flounder.

In conclusion immersion infection, especially immersion and dilution method is an adequate method to induce tenacibaculosis experimentally in Japanese flounder. The method may be a successful means for further investigation on the pathogenesis of this disease and in challenging tests for the development of chemotherapeutic agents and vaccines.

**References**


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