Standardized Method for Experimental Infection of Tiger Puffer *Takifugu rubripes* with Oncomiracidia of *Heterobothrium okamotoi* (Monogenea: Diclidophoridae) with Some Data on the Oncomiracidial Biology

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**ABSTRACT** — 0-year-old tiger puffer, *Takifugu rubripes*, were exposed to suspensions of *Heterobothrium okamotoi* oncomiracidia under different conditions. A fluorescent dye, CFSE (5(6)-carboxy fluorescein diacetate, succinimidyl-ester), was used as a mean of labeling oncomiracidia to find them with a fluorescence microscope immediately after settlement on the host. The labeling procedure did not affect the infectivity of the oncomiracidium. When fish were exposed to labeled oncomiracidia for 1, 3, 5 or 10 h, the settlement rate on the gills increased with increasing exposure time up to 3 h. When similarly exposed to labeled oncomiracidia of different ages (0–4 days after hatching), infectivity of the oncomiracidium decreased rapidly with increasing parasite age, and most oncomiracidia of 2 days or older failed to infect the gills. When exposed to 29, 58 and 116 labeled oncomiracidia/ L/ fish and observed under fluorescence stereomicroscopy immediately after exposure, the settlement rates on the body surface and on the gills were not different among the 3 concentrations tested. The settlement rate on the body surface was always higher than that on the gills, irrespective of the difference in concentration. Thus, infection methods using tiger puffer and *H. okamotoi* oncomiracidia were standardized in terms of exposure time (3 h), parasite age (within 24 h after hatching), and parasite concentration (up to 116 oncomiracidia/ fish). CFSE-staining is suitable for the study of early stages of parasite settlement on the host fish.

**Key words:** *Heterobothrium okamotoi*, oncomiracidium, Monogenea, CFSE, tiger puffer, *Takifugu rubripes*

Many parasites have been reported in or on cultured tiger puffer, *Takifugu rubripes* (see Ogawa and Yokoyama, 1998). Among them, infection with the monogenean *Heterobothrium okamotoi* may be one of the most serious parasitic diseases (Ogawa and Inouye, 1997a). The parasite was tolerant to almost all chemical treatments tested, and the only effective method was to treat infected fish with hydrogen peroxide (Ogawa and Yokoyama, 1998). However, it is practically impossible to eradicate the parasite with a mere repetition of this chemical treatment. Control methods have not yet been established. Biological and ecological knowledge of the parasite should then be efficiently incorporated into the control strategy, but much remains to be studied on the parasite biology.

From the prophylactic point of view, it is crucially important to know how freshly hatched larvae (oncomiracidia) find the host and settle on them. No such data are available on *H. okamotoi*. These aspects will be analyzed through controlled experiments. Thus, the first approach to the study of oncomiracidial biology should be to determine suitable experimental conditions and to establish a standardized method for quantitative infections.

It is generally assumed that oncomiracidia directly reach the habitat, then deciliate and settle there for further growth. There are a few reports about oncomiracidial settlement on the host fish. For example, settlement of the oncomiracidium of a capsalid monoge-
Heterobothrium okamotoi first grows on the gills of the portals of entry into fish (Yokoyama and Urawa, 1986). On the other hand, the oncomiracidium of Discocotyle sagittata, a gill parasite of salmonid fish, did not show any response to host mucus or to the body of the host (Paling, 1969). In Neobenedenia girellae, a capsalid monogenean parasitizing a wide range of marine fish, its oncomiracidium showed settlement activity in vitro in the presence of skin extracts from several species of marine fish (Yoshinaga et al., 2000). Thus, knowledge of oncomiracidial behavior is quite limited, and their behavioral pattern differs substantially among the species studied.

Heterobothrium okamotoi first grows on the gills of tiger puffer, and subsequently migrates to the branchial cavity wall (Ogawa and Inouye, 1997a, b). However, it is not clear whether its oncomiracidium settles selectively on the gills. Since the larva is only 200–300 μm long and lacks pigmented eyes (Ogawa, 1998), it is difficult to find and accurately count the number of oncomiracidia immediately after settlement on the fish. Therefore, we used oncomiracidia labeled with a fluorescent dye, CFSE (5(6)-carboxyfluorescein diacetate, succinimidyl-ester), in quantitative infections. CFSE has been used as a marker in the analysis of cell fate during embryonic development (Paramore et al., 1992). The dye is a lipophilic molecule that is deacetylated by intracellular macromolecules (Fujioka et al., 1994). It is considered to be nontoxic to labeled cells and not to diffuse to adjacent tissues (Paramore et al., 1992). In fish parasitology, actinosporean stages of myxosporeans have been labeled in order to determine the portals of entry into fish (Yokoyama and Urawa, 1997).

In the present paper, some biological aspects of the settlement process of oncomiracidia were studied by standardized experimental infections using the fluorescent labeling technique. Data presented here will provide a basis for further studies of Heterobothrium infection of tiger puffer, including host recognition by the parasite and growth on the host.

Materials and Methods

Experimental conditions

All the in vivo experiments were conducted at 20°C. The salinity of the seawater used was 27‰.

Parasite eggs

Infected, 1-year-old tiger puffer were kept in 500-L aquaria, equipped with external filters (Eheim; Germany). Deposited eggs became entangled on aeration tubes in the aquaria. These eggs were collected with forceps, washed with 0.45 μm-filtered seawater and transferred to tissue culture flasks (50 mL; Iwaki, Co. Ltd., Japan) filled to capacity with filtered seawater. The eggs were incubated at 15°C. Since the infectivity of oncomiracidia may be influenced by the nutritional condition of the host, eggs from many adult parasites were mixed in this study.

Experimental fish

Parasite-free 0-year-old tiger puffer purchased from a hatchery were maintained in large tanks at 20°C, and fed once a day until used. They were 5.0–13.5 cm in body length, when used for experiments. Feeding was stopped one day prior to the start of experiments and no food was given during the experimental periods. In infection experiments, fish density was always kept at 1 individual/L.

Labeling with CFSE

A stock solution of CFSE (Molecular Probes, Inc.) was prepared at 10 mM in 100% dimethylsulfoxide (DMSO) and kept at 5°C until used. For labeling, a CFSE stock solution was added to an oncomiracidial suspension to produce a concentration of 10 μM, mixed with repeated pipetting, and then left for 15 min at room temperature. After labeling, oncomiracidia were collected by filtration with 20 μm nylon meshing and resuspended in filtered seawater.

To examine the retainability of the dye in the parasite, labeled oncomiracidia were maintained in a tissue flask filled with filtered seawater and observed daily for 5 days under a fluorescence stereomicroscope. In addition, as a preliminary experiment, labeled oncomiracidia were exposed to 0-year fish. Gills of several fish were fixed daily with phosphate-buffered 10% formaldehyde and examined for settled parasites. Settled parasites later than 3 days after fixation were detected with difficulty because of reduced fluorescence of the parasites and increased non-specific fluorescence of the gill tissue. Thus, fixed gills were observed under a fluorescence stereomicroscope within 2 days after fixation.

Influence of CFSE on oncomiracidium infectivity

Oncomiracidia hatched within 24 h were divided into two equal aliquots. One was labeled with CFSE and the other left unstained. Infection experiments were done by exposing 10 tiger puffer to 3,000 labeled or non-labeled oncomiracidia in an identical plastic aquarium containing 10 L seawater. After 3 h exposure with sufficient aeration, the two groups of fish were separately maintained in aquaria containing oncomiracidia-free seawater. After 3 days, all fish were fixed in phosphate-buffered 10% formaldehyde and dissected, and then the numbers of parasites on the gills were counted.
Standardized method for *Heterobothrium* infection

**Exposure time**

Oncomiracidia hatched within 24 h were collected and labeled by CFSE. They were transferred to filtered seawater by using 20 µm nylon meshing and counted. Four groups of tiger puffer were exposed to oncomiracidia in four identical plastic aquaria, each containing 5 L seawater, 5 tiger puffer and 210 oncomiracidia. Infection periods of each group were 1 h, 3 h, 5 h and 10 h. After each infection period, fish were anaesthetized with MS-222 and fixed in phosphate-buffered 10% formalin. Subsequently, gills were dissected and the numbers of settled parasites on the gills were counted under a fluorescence stereomicroscope.

**Age of oncomiracidia and infectivity**

Oncomiracidia hatched within 2 h at 15°C were divided into 5 groups for experimental infections. All infections utilized 5 tiger puffer which were exposed to 170 labeled oncomiracidia in 5 L of seawater for 3 h. The first group was used immediately to infect puffers. The other groups of larvae were stored at 15°C and used for infections after 1 day, 2 days, 3 days and 4 days. Immediately after exposure, fish were anaesthetized with MS-222, and then dissected. The number of parasites was counted on the gills using a fluorescence microscope.

**Exposure to different concentrations of parasite larvae and resultant oncomiracidial settlement on host**

Each of 3 groups of 5 tiger puffer was exposed for 3 h to 145, 290 and 580 labeled oncomiracidia, respectively, in 5 L of aerated seawater (Group 1–3). Immediately after exposure, fish were individually anaesthetized with MS-222, and oncomiracidia on the body surface were observed with a fluorescence stereomicroscope. The numbers of oncomiracidia on the following separate regions of the body surface were recorded: head, anterior dorsal body, anterior ventral body, posterior dorsal body, posterior ventral body, pectoral fin, dorsal fin, anal fin, caudal fin (Fig. 1). Subsequently, fish were fixed in phosphate-buffered 10% formalin and then dissected. The number and distribution of settled oncomiracidia on the gills and branchial cavity wall were also recorded within 2 days after fixation. The settlement rate was calculated as the percentage of added oncomiracidia found attached on the fish.

**Results**

**Labeling with CFSE**

Cilia and some internal organs of oncomiracidia were stained bright green (Fig. 2a, b). Fluorescence of labeled oncomiracidia free in the water did not reduce significantly for 5 days. On the other hand, fluorescence of formalin-fixed oncomiracidia settling on the gills was lost quickly. It was only possible to detect parasites until 1 day after exposure (data not shown).

**Influence of CFSE on oncomiracidial infectivity**

Mean intensity of infection with the CFSE-labeled group was 132.8 ± a S. D. of 26.6 parasites per fish, while that with the non-stained group was 135.4 ± 29.8, with no significant difference between the two groups (Mann-Whitney’s U-test; P> 0.05).

**Exposure time**

It was difficult to accurately count the number of oncomiracidia immediately after settlement on the gills with a stereomicroscope with normal illumination (Fig. 3a), but it was quite easy with a fluorescence stereomicroscope (Fig. 3b). When tiger puffer were exposed to oncomiracidia suspended in seawater for 10 h, an average of 56.2 ± a S. D. of 17.0% (an average of 23.6 larvae attached) of added oncomiracidia settled on the gills (Fig. 4). The number of attached oncomiracidia after 10 h exposure was significantly different from that after 1 h exposure (Mann-Whitney’s U-test; P<0.01), but not different from those after 3 h and 5 h exposure (P>0.05).

**Age of oncomiracidium and infectivity**

Immediately after hatching, the settlement rate on the gills was 36% (Fig. 5). As oncomiracidia aged, their infectivity decreased rapidly. Two days after hatching or longer, most oncomiracidia failed to infect the gills, but 0.58% of 4-day-old oncomiracidia maintained infectivity (Fig. 5).

**Exposure to different concentrations of parasite suspensions and resultant oncomiracidial settlement on host**

Oncomiracidia settled both on the body surface (Fig. 6) and on the gills, but no parasite was found attached to the branchial cavity wall. Those on the body surface were deciliated and opened their haptor as on the gills. The number of settled oncomiracidia on the body surface was always higher than those on the gills in the 3
Fig. 2. *Heterobothrium okamotoi* oncomiracidium stained with CFSE (fixed in 10% formalin). a, observed with light microscopy; b, same material as a, observed with fluorescence microscopy. Cilia and some internal organs were stained bright green. Bar = 0.1 mm.

Fig. 3. CFSE-stained *Heterobothrium okamotoi* oncomiracidia (arrowheads) settled on the gills of tiger puffer. a, observed with normal illumination; b, same material as a, observed with fluorescence stereomicroscopy. Eleven oncomiracidia are present. Bar = 0.2 mm.

Fig. 6. Oncomiracidia (arrowheads) settled on the body surface and observed with fluorescence stereomicroscopy. Bar = 3 mm.
Fig. 4. Relationship between the settlement rates of *Heterobothrium okamotoi* oncomiracidia on the gills and exposure time. Settlement rates (%) = (Number of settled oncomiracidia/ Number of oncomiracidia added) × 100. They are expressed as an average ± S. D.

Fig. 5. Relationship between days after hatching of *Heterobothrium okamotoi* oncomiracidia and settlement rates on the gills. Settlement rates are expressed as an average ± S. D.

Forty-two percent of settled oncomiracidia on the body surface were concentrated on the head (Table 1). On the other hand, no preference for a particular gill (tiger puffer has 3 pairs) (Table 2) or for any region of the gills (Fig. 8) was evident.

**Discussion**

For biological studies on the *Heterobothrium* infection of tiger puffer, establishment of a quantitative standardized method is essential to understand the oncomiracidial settlement on the host fish, e.g. to compare the settlement rates in different conditions after exposure. However, such methods have not been developed in monogenean studies. Therefore, at first, experiments were done to determine the basic conditions that may influence the infection.

When fish were exposed for longer than 3 h, the settlement rates on the gills did not increase significantly. Experimental fish were under stressful conditions during exposure, and fish exposed for longer than 3 h were lethargic and secreted a large amount of mucus. Accordingly, the standardized exposure time was set at 3 h.

The oncomiracidium can swim for a maximum of 7

**Table 1.** Number of *Heterobothrium okamotoi* oncomiracidia settled on different regions of the body surface of tiger puffer

<table>
<thead>
<tr>
<th>Body parts:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>28 (45)</td>
<td>7 (11)</td>
<td>12 (19)</td>
<td>9 (15)</td>
<td>4 (6)</td>
<td>2 (3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>62 (100)</td>
</tr>
<tr>
<td>Group 2</td>
<td>58 (43)</td>
<td>45 (33)</td>
<td>14 (10)</td>
<td>10 (7)</td>
<td>3 (2)</td>
<td>4 (3)</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>136 (100)</td>
</tr>
<tr>
<td>Group 3</td>
<td>106 (41)</td>
<td>54 (21)</td>
<td>47 (18)</td>
<td>17 (7)</td>
<td>17 (7)</td>
<td>13 (5)</td>
<td>3 (1)</td>
<td>3 (1)</td>
<td>0 (0)</td>
<td>169 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>192 (42)</td>
<td>106 (23)</td>
<td>73 (16)</td>
<td>36 (8)</td>
<td>24 (5)</td>
<td>19 (4)</td>
<td>4 (0.9)</td>
<td>4 (0.9)</td>
<td>0 (0)</td>
<td>367 (100)</td>
</tr>
</tbody>
</table>

Figures in parentheses represent percentages of oncomiracidia settled on each body region.

Body regions: 1, head; 2, anterior dorsal body; 3, anterior ventral body; 4, posterior dorsal body; 5, posterior ventral body; 6, pectoral fin; 7, dorsal fin; 8, anal fin; 9, caudal fin.
days at 20°C and its life span extends for up to 9 days (Ogawa, 1998). However, the relationship between age and infectivity of the oncomiracidium has never been studied in detail. In the present study, it became clear that infectivity for host gills was reduced sharply with days passed, and that 3 days after hatching most oncomiracidia had lost infectivity. From the practical point of view, the use of oncomiracidia hatched within 24 h is recommended for infection experiments. On the other hand, it is noteworthy that, even 4 days after hatching, some oncomiracidia retained infectivity.

As a result, infection methods were standardized as follows: exposure time 3 h; oncomiracidial age within 24 h after hatching; 29-116 oncomiracidia/ fish. These concentrations did not affect the settlement rate within the range tested.

According to Ogawa and Inouye (1997b), mucus scraped from the body surface of cultured tiger puffer did not contain H. okamotoi. However, because the intensity of infection was low during their regular surveys, and parasites were not stained with fluorescent dye, recently attached parasites might have been overlooked. The oncomiracidium may possibly migrate to the gills from the body surface (Ogawa and Inouye, 1997a). Alternatively, after settlement on the body surface, parasites may eventually be dislodged from the body surface by the water current.

In the present studies, when oncomiracidia settled, they did not distinguish the gill from the body surface. Independent of different concentrations of oncomiracidia, approximately 30% of added oncomiracidia settled on the gills and 40% settled on the body surface and fins. There may have been some differences in the quality of mucus between the gills and body surface, but the oncomiracidium recognized the settlement-inducing substance contained both in the gill mucus and in the skin mucus. Indirect evidence suggests that oncomiracidia of polyopisthocotylean monogeneans reach the gills directly and that they do not attach first on the body surface (Whittington et al., 1999). In this study, by labeling oncomiracidia with CFSE, it was possible to detect settling larvae in situ on the host immediately after exposure of oncomiracidia to fish. It is highly desirable to apply this labeling technique to oncomiracidia of other polyopisthocotyleans to discover whether attachment on the body surface of the host is a special feature of H. okamotoi or more widespread.

Tiger puffer stay under the sediment, especially at night time, with their eyes, part of dorsal surface and dorsal fins exposed to the water (Fujita, 1988). It is

<table>
<thead>
<tr>
<th>Group</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>First gill</td>
<td>10 (26)</td>
<td>24 (30)</td>
<td>83 (38)</td>
</tr>
<tr>
<td>Second gill</td>
<td>12 (30)</td>
<td>25 (31)</td>
<td>58 (26)</td>
</tr>
<tr>
<td>Third gill</td>
<td>17 (44)</td>
<td>31 (39)</td>
<td>79 (36)</td>
</tr>
</tbody>
</table>

Figures in parentheses represent percentages of oncomiracidia settled on each gill.

29 larvae/ fish  58 larvae/ fish  116 larvae/ fish

Fig. 8. Distribution of settled Heterobothrium okamotoi oncomiracidia on the gills of tiger puffer immediately after 3 h exposure to oncomiracidial suspensions of different concentrations. Each dot represents a settled parasite. Parasites recovered on the 3 sets of gills of the 5 fish in each experimental group were pooled and shown in one figure.
assumed that most *H. okamotoi* oncomiracidia reach the gills passively through the respiratory currents created by the host. On the other hand, the body surface is well protected from oncomiracidial settlement by the surrounding sand. If this is the major way of infection for the oncomiracidium in natural waters, the parasite might not have developed a special route of invasion from the body surface. It is important to know the fate of oncomiracidia of *H. okamotoi* attached on the body surface.

The number of oncomiracidia settling on the head or dorsal body surface was considerably higher than that on the ventral body surface. The oncomiracidium of *H. okamotoi* swims in a downward direction (Ogawa, unpublished data). This may be the major reason for this biased settlement pattern on the host. This swimming characteristic of the oncomiracidium is probably favorable to the parasite, since positive geotaxis may enhance the chances of the parasite encountering the host fish, which leads a benthic life in the natural environment (Fujita, 1988).

Parasites on the gills were located on the basal region of the gill filaments at 7 days after exposure, and later moved to the distal region (Ogawa and Inouye, 1997c). In the present experiments, however, this movement was not detected. There are two possible interpretations of these different results. First, in the experiments of Ogawa and Inouye (1997c) they may have migrated from the original locations to the basal region of the gill filaments within 7 days of settlement. Secondly, in the present experiment, 0-year-old fish were used, while Ogawa and Inouye used 1-year-old fish. Difference in fish size might cause different results. It is likely that the respiratory water current of the larger fishes was stronger than that of the smaller fishes, and they may have led to a concentration of larvae on the basal region of the gill filaments.

After settlement of the oncomiracidium on the host, fluorescence of CFSE was lost rapidly. Therefore, this dye is not suitable for use in long-term experiments. On the other hand, because oncomiracidia in water keep their fluorescence for at least 5 days, it can be used for experiments before they start feeding from the host. When the oncomiracidia settle on the host and start feeding, their metabolism is activated and the dye may disappear rapidly. However, because CFSE does not influence the infectivity of oncomiracidium, it may be a useful tool for selected monogenean studies.

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References


