Seawater Survival of *Enteromyxum leei* (Myxozoa) Evaluated by *In Vitro* Viability and *In Vivo* Infectivity Assays

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(Received July 6, 2009)

ABSTRACT—A vital staining assay using Hoechst 33342 and propidium iodide was developed for the determination of viability of *Enteromyxum leei* (Myxozoa), a causative agent of myxosporean emaciation disease. Using this *in vitro* viability assay combined with *in vivo* infectivity test, the survival of developmental stages of *E. leei* in seawater was evaluated. Developmental stages of *E. leei* were freshly isolated from infected tiger puffer *Takifugu rubripes* and incubated in filtered natural seawater at 20°C for 0, 6, 12 and 24 h. The *in vitro* staining assay showed a time-dependent decrease in viability, and no viable stages were detected at 24 h. In the first trial of *in vivo* infectivity, naïve tiger puffer were exposed to *E. leei* suspensions which were incubated in seawater for 0–48 h. In the second trial, naïve grass puffer *T. niphobles* were fed *E. leei* incubated for 0–24 h. After rearing for 3–4 weeks, infection with *E. leei* was detected in fish challenged with the parasite incubated for 24 and 6 h in the first and second trials, respectively. Results of the *in vitro* and *in vivo* assays showed that the survival time of developmental stages of *E. leei* in seawater was variable but estimated less than 24 h.

Key words: *Enteromyxum leei*, Myxozoa, Hoechst 33342, propidium iodide, myxosporean emaciation disease, *Takifugu rubripes*, tiger puffer

Enteromyxum leei is an enteric parasite that causes the myxosporean emaciation disease of cultured marine fishes, in particular tiger puffer *Takifugu rubripes* in Japan (Tin Tun *et al.*, 2000; Yanagida *et al.*, 2004; Ishimatsu *et al.*, 2007). Recently, the host range of the parasite was extended to other fishes including Japanese flounder *Paralichthys olivaceus*, red sea bream *Pagrus major* and spotted knifejaw *Oplegnathus punctatus* in Japanese aquaculture (Yasuda *et al.*, 2005; Yanagida *et al.*, 2008). Although the life cycle of myxosporeans generally involves the actinosporean stage in the alternate annelid hosts (Yokoyama, 2003), fish-to-fish transmission of *E. leei* was experimentally demonstrated (Diamant, 1997; Yasuda *et al.*, 2002). It has been considered that infected fish excretes gut mucosa fragments containing numerous developmental stages of *E. leei*, causing oral transmission to other fish (Diamant, 1997). It is probable that this unique characteristic of the parasite results in such a rapid spread of the disease within/between sea cages. The pre-sporogonic developmental stages of *E. leei* are believed to be infectious to other fish, but the biology of the infective

stage, e.g., the survival of the parasite in seawater, is poorly understood. The viability of infectious developmental stages is likely to have a considerable impact on the parasite's dispersion in fish farms.

Until now, a reliable method for determining the viability of myxosporeans has not been fully developed. Dye-exclusion assays using eosin, eosin-nigrosin and trypan blue were documented as a viability test for developmental stages of *Enteromyxum* spp. (Redondo *et al.*, 2003; Yokoyama and Shirakashi, 2007). However, these methods often cause variable and inconsistent results. A dual staining with fluorescein diacetate (FDA) and propidium iodide (PI) was also utilized for assessment of myxosporean and actinosporean viability (Markiw, 1992; Yokoyama *et al.*, 1997). FDA is deacetylated by intracellular esterase to yield green fluorescence in viable cells, while PI permeates only through the membrane of dead cells and emits a red fluorescence. Using the FDA-PI staining, viable (stained green) and non-viable (stained red) cells were distinctly visualized by fluorescent microscopy. However, a problem of relatively rapid fading of green fluorescence by FDA remained to be solved. Another disadvantage of this method is a difficulty in discriminating the developmental stages of *E. leei* from host epithelial

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cells in wet preparation.

The fluorescent dye Hoechst 33342 (HO) penetrates the membrane of both live and dead cells, specifically binds nucleic acid and intercalates into DNA, resulting in emission of bright blue fluorescence by ultraviolet light. HO stain has been commonly used for cell viability in combination with PI (Foglieni *et al.*, 2001; Rowland *et al.*, 2003; Cai *et al.*, 2005). Double staining with HO and PI discriminates accurately between live and dead cells because PI quenches the HO fluorescence of dead cells (Cai *et al.*, 2005). Our preliminary experiment also revealed that HO stain allowed distinguishing the developmental stages of *E. leei* from the host cells on the basis of the multinuclear characteristics of the myxosporean plasmodia. The objective of the present study was to develop a vital staining technique with HO and PI for determining the viability of *E. leei*, and to evaluate the survival of developmental stages of *E. leei* in seawater using the *in vitro* viability and *in vivo* infectivity assays.

Materials and Methods

Vital staining with HO and PI

As a source of *E. leei* material, three experimentally infected tiger puffer (average body length = 8.3 cm, average body weight = 22.4 g) were used. Fish were dissected, and the intestinal mucosa was scraped using a sterile razor blade. Developmental stages of *E. leei* were collected in physiological saline, passed through a stainless steel mesh screen ('40 mesh screen' with opening size of 380 μm , Cell Dissociation Sieve, Sigma) and centrifuged at $365 \times g$ for 10 min. After the supernatant was discarded, the pellet was divided into two parts. One part was suspended in physiological saline, while the other was fixed in 10% formalin (as a negative control). Working solutions of HO (1 mM dissolved in phosphate buffered saline (PBS), bisbenzimidazole H33342 trihydrochloride, Sigma) and PI (0.02 mg/mL dissolved in PBS, Sigma) were prepared. Twenty five μL of parasite suspension, 25 μL of PI solution and 50 μL of HO solution were mixed in a 1.5 mL microtube and kept in the dark at 5°C for 15 min. After a cover slip was placed over a drop of 25 μL of the mixture on a glass slide, parasites were observed with an ultraviolet epifluorescent microscope (BX60, Olympus). On the basis of 50 multinuclear developmental stages of *E. leei*, the fluorescence pattern was classified as follows; 1) only nuclei showing blue fluorescence (HO-positive, PI-negative), 2) mixed nuclei showing blue or red fluorescence (HO-positive, PI-positive or negative), 3) only nuclei showing red fluorescence (HO-positive, PI-positive) (Fig. 1).

In vitro viability assay of *E. leei* incubated in seawater

Developmental stages of *E. leei* were isolated from

Fig. 1. Three fluorescence patterns of developmental stages of *Enteromyxum leei* stained with Hoechst 33342 (HO) and propidium iodide (PI) observed under UV light (**a**: HO +, PI –; **b**: HO +, PI +/-; **c**: HO +, PI +). Arrows and arrowheads show HO-positive and PI-positive nuclei, respectively. Bar = 10 μm .

the intestinal mucosa of seven experimentally infected tiger puffer (average body length = 8.7 cm, average body weight = 24.2 g). After the suspension of *E. leei* was prepared as described above, the parasite material was divided equally into five tubes containing 10 mL filtered natural seawater (ca. 32‰) supplemented with an antibiotic mixture (100 units/mL penicillin G, 100 $\mu\text{g/mL}$ streptomycin, 0.25 $\mu\text{g/mL}$ amphotericin B). The five tubes were kept at 20°C in the dark. After 0 (positive control), 6, 12 and 24 h, each tube was centrifuged at $365 \times g$ for 10 min. The supernatant was removed,

and the pellet was resuspended in 1 mL of PBS. Parasite density was estimated by hemacytometer, followed by the vital staining with HO and PI. Fluorescence pattern of parasites ($n = 50$) was classified into three groups as described above by fluorescent microscopy. Parasites with only nuclei showing bright blue fluorescence (HO +, PI –) were defined as viable, and parasite viability (%) was estimated on the basis of 50 developmental stages of *E. leei*.

First trial of in vivo infectivity

Eight experimentally infected tiger puffer (average body weight = 201.3 g) were used as 'donor' fish. Developmental stages of *E. leei* were collected from the intestine and suspended in 5 L of filtered natural seawater (ca. 32‰) at 20°C. After 0 (positive control), 6, 12, 24 and 48 h, each 1 L of the parasite suspension was mixed with 100 L seawater to prepare as 'infective water'. Each 20 'recipient' naïve tiger puffer (average body weight = 30.2 g) were exposed to the 'infective water' in a 100 L tank (ca. 27°C) provided with aeration. After 6 h immersion, the fish were split into two tanks (500 L of water volume, ten fish each) supplied with sand-filtered seawater and reared at $26.8 \pm 4^\circ\text{C}$. During the experimental period, all fish were fed a commercial pellet diet at about 0.5% of body weight/day. Unexposed fish ($n = 20$) were held under equivalent conditions as a negative control. After 3 weeks, all surviving fish were dissected and the gut tissues were excised. Parasites were detected by microscopic observation of Diff-Quik stained imprint slides of the intestinal mucosa, as well as PCR-based assay of 80% ethanol-fixed gut tissues as described in Yanagida *et al.* (2005).

Second trial of in vivo infectivity combined with in vitro assay

In the second experiment, *in vitro* viability and *in vivo* infectivity assays were used in parallel to evaluate the survivability of *E. leei* at several time points within 24 h of incubation in seawater. Tissues infected with *E. leei* were obtained from 20 experimentally infected tiger puffer (average body length = 12.3 cm, average body weight = 47.4 g), and developmental stages of the parasite were harvested by filtration through a stainless steel mesh to remove the tissue debris. The parasite suspension was divided equally into four tubes contain-

ing 10 mL of filtered natural seawater with an antibiotic mixture (100 units/mL penicillin G, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B) and kept at 20°C in the dark. After 0 (positive control), 6, 12 and 24 h, the parasites were concentrated into 1 mL of PBS by centrifugation at $365 \times g$ for 10 min. Parasites were enumerated using hematocyte counting chambers, and the parasite viability was determined by the *in vitro* viability assay as described above.

Naïve grass puffer *Takifugu niphobles* (average body weight = 2.0 g) artificially bred by Fisheries Laboratory of The University of Tokyo were used as recipient fish, as their susceptibility to *E. leei* is similar to tiger puffer (Yokoyama and Shirakashi, 2007). Four groups of 15 grass puffer were kept in 65 L tanks (55 L of water volume) equipped with a closed re-circulating filtration system. At the same time points of incubation of *E. leei* described above, 15 grass puffer were fed the sub-samples of incubated parasites mixed with 0.5 g of commercial kneaded feed for eel. As a negative control, 15 grass puffer were fed 0.5 g of commercial kneaded feed mixed with sterilized distilled water. Subsequently, the experimental fish were reared at a constant temperature of 25°C. After 4 weeks, infection with *E. leei* was determined by microscopy and PCR as described in the first trial.

Results

Vital staining with HO and PI

Developmental stages of freshly obtained *E. leei* exhibited the three patterns of fluorescence; 1) HO +, PI –; 2) HO +, PI +/-; 3) HO +, PI +, with percentages of 68%, 24% and 8%, respectively (Table 1). For formalin-fixed *E. leei*, the percentages were 0%, 10% and 90%, respectively. Thus, parasites showing HO + and PI – were considered viable.

In vitro viability assay

Although no considerable change in the parasite density occurred during the period kept in seawater ($1.2\text{--}2.1 \times 10^5$ parasite/mL), degeneration of parasites appeared to progress with time (data not shown). *In vitro* staining assay showed a time-dependent decrease in viability, and no viable stages were observed at 24 h (Table 2). At the end of the experiment (24 h), all nuclei of the parasites stained red (HO +, PI +).

Table 1. Fluorescence patterns of fresh and fixed *Enteromyxum leei* by *in vitro* staining with Hoechst 33342 (HO) and propidium iodide (PI)

Parasite condition	Parasite density (parasites/mL)	Fluorescence pattern (%)		
		HO +, PI –	HO +, PI +/-	HO +, PI +
Fresh	3.4×10^5	68 (± 6.0)*	24 (± 5.1)	8 (± 2.0)
Formalin-fixed	2.8×10^5	0 (± 0)	10 (± 2.5)	90 (± 2.5)

* Values in parentheses show the confidence intervals at 95% in reliability.

Table 2. Viability of *Enteromyxum leei* incubated for 0–24 h in seawater at 20°C and evaluated by *in vitro* staining assay with Hoechst 33342 (HO) and propidium iodide (PI)

Time (h) in seawater	Parasite density (parasites/mL)	Viability (%)	Fluorescence pattern (%)		
			HO +, PI –	HO +, PI +/-	HO +, PI +
0	2.1×10^5	30 (± 5.8)*	30 (± 5.8)	48 (± 6.9)	22 (± 4.8)
6	1.2×10^5	18 (± 4.1)	18 (± 4.1)	52 (± 6.9)	30 (± 5.8)
12	1.4×10^5	6 (± 1.6)	6 (± 1.6)	36 (± 6.4)	58 (± 6.7)
24	1.3×10^5	0 (± 0)	0 (± 0)	0 (± 0)	100 (± 0)

* Values in parentheses show the confidence intervals at 95% in reliability.

First trial of *in vivo* infectivity

Differences between the duplicate tanks were not great enough to exclude the possibility that the differences were due to the tank effect (Fisher's exact test, $p < 0.05$). Accordingly, data from the duplicates were pooled for each time point. Fish exposed to freshly collected (non-incubated) *E. leei* had 15.8% infection prevalence (Table 3). Prevalence of infection decreased with time in seawater down to 0% in 12 and 48 h groups, but 23.5% of fish were infected in 24 h group. Negative controls remained uninfected. Although some of the fish died during the experimental period, no disease signs were observed in any of the groups.

Table 3. Infectivity of *Enteromyxum leei* incubated for 0–48 h in seawater and evaluated by *in vivo* infection experiment using tiger puffer

Time (h) in seawater	Prevalence of infection (%)
0	15.8 (19)*
6	11.1 (18)
12	0 (17)
24	23.5 (17)
48	0 (20)

* Number in parenthesis is the number of fish examined.

Second trial of *in vivo* infectivity combined with *in vitro* viability assay

The parasite density was 1.3×10^6 parasites/mL at the beginning and decreased gradually with time (Table 4). Although 52% of parasites were viable at the initial time, viability rapidly declined to 4% at 6 h. Prevalence of infection in grass puffer fed with fresh (non-preserved) and 6 h preserved *E. leei* was 100% and 60%,

respectively (Table 4). In 12 and 24 h preserved groups, neither viable stages nor infection were observed in *in vitro* and *in vivo* assays, respectively. Negative controls remained uninfected.

Discussion

The present study indicates that a double staining with HO and PI is a possible indicator of the viability of developmental stages of *E. leei*. It may be confusing that some of parasites have exhibited the ambiguous fluorescence pattern (HO +, PI +/-). However, the occurrence of this pattern in formalin-killed parasites demonstrates that HO +, PI +/- *E. leei* are non-viable. Additionally, in the second infectivity trial, regardless of a moderate percentage (32%) of this fluorescence pattern in 12 h incubated parasites, infection did not occur in the parallel exposure group. Thus, it seems to be reasonable to conclude that only *E. leei* exhibiting HO + and PI – are considered viable and infective. Results of the present study also indicate that 'viability' by *in vitro* vital staining corresponds well to 'infectivity' evaluated by the *in vivo* experiment as shown in Table 4. An *in vitro* viability assay would be valuable for the studies on parasite biology and drug sensitivity tests against *E. leei*. Also, if the dyes could penetrate the spore valves of myxosporean, the present staining technique would be likely useful for other myxosporean species.

Variable results were obtained in the initial viability of *E. leei* and the prevalence of infection among the different experiments in the current study. Even though the parasite was freshly collected, some stages might have been deteriorated by host's digestive enzymes or by the process of harvesting, possibly causing the wide

Table 4. Viability of *Enteromyxum leei* incubated for 0–24 h in seawater at 20°C by *in vitro* staining assay using Hoechst 33342 (HO) and propidium iodide (PI), and the infectivity of the parasite at the same time point by *in vivo* infection experiment using grass puffer

Time (h) in seawater	Parasite density (parasites/mL)	Viability (%)	Fluorescence pattern (%)			Prevalence of infection (%)
			HO +, PI –	HO +, PI +/-	HO +, PI +	
0	1.3×10^6	52 (± 6.9)*	52 (± 6.9)	44 (± 6.8)	4 (± 1.1)	100
6	9.1×10^5	4 (± 1.1)	4 (± 1.1)	46 (± 6.9)	50 (± 6.9)	60
12	5.9×10^5	0 (± 0)	0 (± 0)	32 (± 6.0)	68 (± 6.0)	0
24	3.3×10^5	0 (± 0)	0 (± 0)	0 (± 0)	100 (± 0)	0

* Values in parentheses show the confidence intervals at 95% in reliability.

variation in initial viability of *E. leei*. It suggests that the same lot of *E. leei* infected tissue should be used for *in vitro* and *in vivo* studies. Prevalence of infection with *E. leei* in recipient fish varied between the two trials in this study. It is unknown whether this variation was caused by differences in the initial condition of parasite, because the viability of *E. leei* was not determined in the first trial. Alternatively, it may be due to the difference in challenge method (immersion vs *per os* for the first and second trials, respectively). It is likely that recipient fish given *per os* would ingest actively the parasite, resulting in more efficient transmission of *E. leei* than the immersion method. Redondo *et al.* (2002) also found that oral ingestion of infected intestine is the most effective for transmission of *E. scophthalmi*, compared with cohabitation with infected fish or waterborne exposure to the effluent of a tank containing infected fish. To standardize the infection experiment of *E. leei*, further studies are required on the collection of parasite, parasite dose and route of infection.

Overall results of the present study seem to indicate that the survival time of *E. leei* is less than 24 h at 20°C in the marine environment, though it is possible that the survivability is influenced by water temperature. Temperature of parasite incubation was set only at 20°C in the present study, because occurrence of the disease was usually found around 20°C in fish farms, and also because 20°C seemed to be the appropriate temperature for development of *E. leei* (Yanagida *et al.*, 2006). This estimation is comparable to the result of Redondo *et al.* (2003) who reported that developmental stages of *E. scophthalmi* remained viable *in vitro* for approximately one day in seawater at 20°C, though they did not investigate the infectivity of incubated parasites by *in vivo* infection. Nevertheless, in the present study, 23.5% of fish exposed to 24 h incubated *E. leei* were infected in the first trial, while in the second trial no infection was observed in the equivalent group. This difference may be explained by the difference in preparation methods between the two trials. In the second trial, parasite was carefully purified using filtration with stainless steel mesh to remove the host tissue debris, but such a treatment was not done in the first trial. The mucosal remnants derived from the infected intestine might have protected the developmental stages of *E. leei* from osmotic shock (Diamant, 1997; Redondo *et al.*, 2002). However, the data from the first trial may reflect the actual situation in fish farms. Indeed, according to empirical observations, floating mucosal fragments possibly harboring *E. leei* have been frequently found on the water surface of infected cages/tanks.

As a management strategy for preventing the spread of *E. leei*, it is recommended that naïve fish stocks should be kept from infected fish cages as far as possible (theoretically, more than a distance that *E.*

leei migrates within 24 h). However, it is difficult to determine the distance required between neighboring sea cages, because dispersal of the non-motile developmental stages of *E. leei* could be strongly influenced by water currents. Chambers and Ernst (2005) investigated the influence of tidal currents on dispersal of eggs of the monogenean *Benedenia seriolae* in a large-scale farm of kingfish *Seriola lalandi* located in South Australia. Parasite eggs dispersed only at sites along the tidal current, rather than across the current direction. Also, eggs were found to reach a cage placed more than 8 km from the source farm. It would be difficult to apply their data directly to the Japanese farm condition, because fish farms in Japan are densely located within bays where the tidal current is complicated. Evacuation of sea cages to offshore area with faster water flow may be practiced so that the parasite density can be diluted, possibly decreasing the chance of ingestion by susceptible fish. In case of land-based culture farms, increased exchange rates of rearing water would help to flush the waterborne parasite out of fish tank.

Acknowledgements

We would like to thank Mr. Yoshinori Nomura of the Kumamoto Prefectural Fisheries Research Center for his supports in our experiment, Miyazaki Prefectural Fisheries Experimental Station for providing the parasite materials and Fisheries Laboratory of the University of Tokyo for providing the experimental fish.

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