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Disinfection of fertilized eggs of the edible ascidian *Halocynthia roretzi* for prevention of soft tunic syndrome

Akira Kumagai[1,*], Toru Tanabe[1], Akatsuki Nawata[2], Atsushi Suto[2]

[1]Miyagi Prefecture Fisheries Technology Institute, Kesennuma Fisheries Experimental Station, Kesennuma, Miyagi 988-0181, Japan

[2]Miyagi Prefecture Fisheries Technology Institute, Ishinomaki, Miyagi 986-2135, Japan

ABSTRACT: *Azumiobodo hoyamushi*, the causative agent of soft tunic syndrome, was likely introduced to farming sites of the edible ascidian *Halocynthia roretzi* via ascidian spat. The source of infection is thought to be cysts of *A. hoyamushi* that reside in the substrates on which the ascidian spat are attached, but not the spat themselves. Thus, there is a need to develop methods to prevent contamination of the substrates with *A. hoyamushi* during seed production of the ascidian. We evaluated the protozoacidal effects of sodium hypochlorite and povidone-iodine against the flagellate and temporary cyst forms of *A. hoyamushi.* Additionally, we evaluated the effects of these disinfectants on the development of fertilized ascidian eggs. The flagellate form of *A. hoyamushi* was completely inactivated by povidone-iodine (5 ppm, 1 min) and sodium hypochlorite (1 ppm, 1 min). The temporary cysts of *A. hoyamushi* were completely inactivated by both disinfectants (5 ppm, 1 min). Disinfection with 50 ppm povidone-iodine for 15 min or 5 ppm sodium hypochlorite for 15 min had no effect on ascidian embryogenesis. Thus, horizontal transmission of *A. hoyamushi* via the substrates can be efficiently prevented by disinfecting ascidian eggs or tools used for spawning with povidone-iodine baths ranging from 5 ppm for 1 min to 50 ppm for 15 min without any side effects.

KEY WORDS: *Azumiobodo hoyamushi* · Soft tunic syndrome · Povidone-iodine · Sodium hypochlorite · *Halocynthia roretzi* · Egg disinfection

INTRODUCTION

Mass mortality of the cultured ascidian *Halocynthia roretzi* ('sea pineapple') caused by soft tunic syndrome has resulted in significant economic losses in the ascidian aquaculture industry in Korea and Japan since 1995 and 2006, respectively (Jung et al. 2001, Kumagai et al. 2010). The flagellate protozoan *Azumiobodo hoyamushi* (Kinetoplastea: Neobodonida) was isolated from diseased cultured ascidians in Japan and Korea and identified as the causative agent of soft tunic syndrome (Kumagai et al. 2011, Hirose et al. 2012, Kim et al. 2014). *A. hoyamushi* has 2 forms during its life cycle. The flagellate in the tunic of diseased ascidians or in subculture maintenance medium consists of a fusiform cell that possesses 2 flagella and exhibits rapid locomotory behavior. Under adverse conditions, the flagellate transforms to a temporary cyst and adheres to organic and inorganic substrates (Nawata et al. 2015).

In Japan, the geographic area of infection gradually expanded after the initial outbreaks in 2007, and encompassed the majority of aquaculture sites by 2010, suggesting that the flagellate has high infectivity (Kumagai et al. 2010). Since the tsunami caused by the Great East Japan Earthquake destroyed all

*Corresponding author: kumagai-ak557@pref.miyagi.jp

ascidian farming facilities in 2011, there have been no signs of an outbreak of the disease to date in aquaculture facilities in Japan. However, flagellates have been detected in wild *H. roretzi* (Kumagai et al. 2013). Additionally, the stalked sea squirt *Styela clava*, a species related to *H. roretzi* that inhabits the coasts of Japan, is also a potential carrier of *A. hoyamushi* (Kumagai et al. 2014).

The first outbreaks of the disease occurred in Japan in 2007 at farming sites where Korean spat had been introduced, suggesting that *A. hoyamushi* was likely introduced to Japan via Korean spat (Kumagai et al. 2010). In a recent study, Hirose et al. (2014) noted that spat of *H. roretzi* may not be direct carriers of the pathogens, and the infection source may instead be cysts of *A. hoyamushi* that reside in the substrates (e.g. cords of palm fibre) to which the spat attach, because ascidian spats are not susceptible to *A. hoyamushi* in experimental infection. In Japan, the ascidian aquaculture industry typically relies on natural spat collected offshore (Kumagai et al. 2010). As a result, there is a risk that the substrates for larval attachment include cysts of *A. hoyamushi* derived from infected wild spawners. Additionally, the use of hatchery-based seed production of ascidians would not eliminate the risk posed by using brood stock that are asymptomatic carriers of the pathogen.

To prevent disease transmission, the salmonid aquaculture industry has instituted egg-disinfection procedures, using povidone-iodine, prior to the introduction of eggs into hatcheries (Yoshimizu 2009). These egg-disinfection procedures are effective, except for treatment of intra-ovum infections in which the pathogen has already entered the eggs. Such infections include bacterial kidney disease (Evelyn et al. 1984) and bacterial cold-water disease (Brown et al. 1997, Kumagai & Nawata, 2010), but to our knowledge, they do not include diseases caused by flagellates.

Thus, we hypothesized that the application of disinfection procedures to eggs and tools used for spawning (e.g. mesh nets for collecting eggs), prior to the introduction of eggs into the hatching tank in which the larva settle on the substrates, would prevent contamination of substrates with *A. hoyamushi*, even if the broodstock for artificial seed production included a carrier of *A. hoyamushi*. Thus, use of the disinfection procedures would prevent horizontal transmission of *A. hoyamushi* to adult ascidians in farming areas that rely on spat introductions.

We evaluated the effects of 2 disinfectants, viz. sodium hypochlorite and povidone-iodine, on the development of fertilized ascidian eggs and documented the disinfectant activity of these disinfectants against *A. hoyamushi* at 2 life stages, i.e. the flagellate form and the temporary cyst form.

MATERIALS AND METHODS

Influence of disinfection on the development of ascidian fertilized eggs

We obtained 2 yr old healthy mature ascidians (n = 20, 150 g body weight) from an aquaculture site in Miyagi Prefecture, Japan, where the disease had not occurred previously. The ascidians were reared in a 100 l aquarium at the Miyagi Prefecture Fisheries Technology Institute. The experimental aquarium was supplied with seawater that was pumped from offshore of the laboratory and filtered through a sand filter. Fertilized eggs were collected using an 80 µm nylon mesh net within 2 h after the occurrence of natural spawning in the aquarium containing 20 mature ascidians. The eggs were rinsed with 5 l of seawater that was filtered through a 2 µm filter to remove excess milt. Stereomicroscopic observation confirmed that cell division had not started.

The fertilized eggs (n ≈ 100 000) were divided into 15 groups, each consisting of 5000 eggs. Each group was immersed in 1 l of either a sodium hypochlorite solution (12%, 30, 20, 10, or 5 ppm; Iwaki Seiyaku) or seawater (control) for 30 min (3 groups treatment^{-1}). The sodium hypochlorite solution was diluted with filtered seawater (2 µm filter). After each disinfected egg group was rinsed with the filtered seawater, half the eggs in each group (n = 2500) were incubated at 10°C in a 500 ml beaker filled with static, aerated filtered seawater for 7 d.

The disinfected eggs were observed to determine survival at 4 h and 2 and 7 d after spawning. In the fourth hour after spawning, approximately 200 eggs were sampled from each group and observed under a stereomicroscope (10×) to determine the proportion of fertilized eggs that underwent cleavage. Similarly, 2 d after spawning, approximately 200 eggs from each group were examined to estimate the proportion of fertilized eggs that successfully hatched. Subsequently, tadpole-like larvae including eggs not undergoing cell division (n = 200 in total) were transferred to a 40 ml petri dish and incubated at 10°C until 7 d after spawning. At this time, we examined each dish using a stereomicroscope to estimate the proportion of fertilized eggs that had developed to the settled larval stage.

Two days later, we evaluated the effect of povidone-iodine (Isodine, Meiji Seiyaku) at 2 different concentrations (50 and 25 ppm) on the development of fertilized ascidian eggs using the method described above. The temperature of the seawater in which adult ascidians were reared ranged from 8 to 10°C. All proportion data were normalized using an arcsine square root transformation and subsequently analyzed using 1-way analysis of variance and Dunnett's multiple comparison test.

In vitro disinfectant activity against *Azumiobodo hoyamushi*

Flagellate form

We obtained a spontaneously infected ascidian (1 yr old, 95 g body weight) from a farming site in Miyagi Prefecture. The softened tunic (25 g) of the infected ascidian was cut into small pieces (5 × 1 cm) and then incubated in 200 ml of sterilized seawater at 15°C for 4 h. After incubation, the seawater was passed through a 1 µm nylon mesh (NYTAL, SEFAR) to remove other organisms to the greatest extent possible. The concentration of the flagellate cell suspension was determined using a Burker–Turk hemocytometer.

The flagellate cell suspension (2.0×10^5 cells ml^{-1}) was transferred to a 24-well microtitration plate (Iwaki Microplate; Asahi Glass) at 1 ml volume $well^{-1}$. Subsequently, we added 0, 2.5, or 5 µl of 100 ppm sodium hypochlorite solution (in seawater) or 1, 2, 3, 4, or 5 µl of 1000 ppm sodium hypochlorite solution (in seawater) to the flagellate cell suspension. The final concentrations of sodium hypochlorite in the mixtures were 0, 0.25, 0.5, 1, 2, 3, 4, or 5 ppm. The mixtures were incubated for 1, 5, 10, or 15 min at 15°C, and then 20 µl of each mixture was observed under a light microscope (100×) to check for changes in the motility of the flagellates. Motility was classified into 1 of 4 groups using the modified criterion of Park et al. (2014) (see Table 1). The experiment was repeated using povidone-iodine solution in place of sodium hypochlorite.

Temporary cysts

The flagellate was subcultured in 50 ml of maintenance medium at 15°C for 7 d following the method described by Kumagai et al. (2011). After subculture, 25 ml of the flagellate cell suspension (4.0×10^6 cells ml^{-1}) was centrifuged at 500 × *g* (5 min at 10°C). The supernatant was discarded and the pellet was re-suspended in 25 ml of sterilized artificial seawater (SASW). This series of steps was repeated twice, and the pellet was re-suspended in 120 ml SASW. A 2 ml aliquot of the flagellate cell suspension (2.8×10^5 cells ml^{-1}) was pipetted into each well of a 12-well microtitration plate (Iwaki Microplate; Asahi Glass). After incubation for 2 d at 15°C to induce transformation to a temporary cyst, the SASW was discarded and the wells were washed 5 times with SASW to remove swimming flagellates. After the SASW was discarded, 2 ml of either 5 ppm povidone-iodine solution (diluted in SASW) or 5 ppm sodium hypochlorite solution (diluted in SASW) was pipetted into a single well. After incubation for 1 or 5 min at room temperature, the disinfectants were removed and the well was washed with SASW. We used SASW in place of the disinfectant in the controls. The numbers of temporary cysts adhering to the bottoms of the wells were counted in 10 viewing fields using an inverted microscope, both before and after incubation with the disinfectants. We subsequently added 1 ml of tunic extract to each plate well to induce excystment of the flagellate. After 1 h, we counted the numbers of temporary cysts and confirmed induction of excystment in the flagellate (appearance of swimming flagellates) in each well using an inverted microscope. The tunic extract consisted of the filtrate (0.45 µm) from 90 ml of SASW that was incubated with small pieces of tunics (18 g) from healthy ascidians for 1 d at 15°C. Induction of encystment in seawater and excystment with the tunic extract were performed according to the method described by Nawata et al. (2015).

RESULTS

Influence of disinfection on the development of ascidian fertilized eggs

Among the disinfected groups treated with sodium hypochlorite solution, the ratio of cleaved eggs to fertilized eggs was significantly lower (Dunnett's test, $p < 0.05$) at 10 ppm sodium hypochlorite than in the control group. The ratio of hatched larvae to fertilized eggs was significantly lower ($p < 0.01$) at 30 ppm sodium hypochlorite than in the control group. The ratio of settled larvae to fertilized eggs was significantly lower ($p < 0.01$) at 20 ppm than in the control group (Fig. 1). Disinfection with povidone-iodine solution (25 and 50 ppm) had no effect on develop-

ment of fertilized eggs. There was no significant difference between disinfected groups and the control at any developmental stage (Dunnett's test, $p > 0.05$: Fig. 2).

In vitro disinfectant activity against *Azumiobodo hoyamushi*

Flagellate form

Sodium hypochlorite completely killed the flagellate form of *A. hoyamushi* (2.0×10^5 cells ml^{-1}) at a concentration of 1 ppm in 1 min (Table 1). Povidone-iodine solutions completely inactivated the flagellate form of *A. hoyamushi* at an available iodine concentration of 5 ppm in 1 min (Table 1).

Temporary cysts

Povidone-iodine and sodium hypochlorite solutions completely inhibited excystment of temporary cysts of *A. hoyamushi* at an available concentration of 5 ppm in 1 min. There were no swimming flagellates in these wells. In contrast, 80% of the temporary cysts of *A. hoyamushi* transformed to the flagellate form in the control and resumed swimming in the well (Table 2).

DISCUSSION

Egg disinfection using 50 ppm of povidone-iodine for 15 min or 5 ppm of sodium hypochlorite for 15 min had no effect on development (cleavage, hatching,

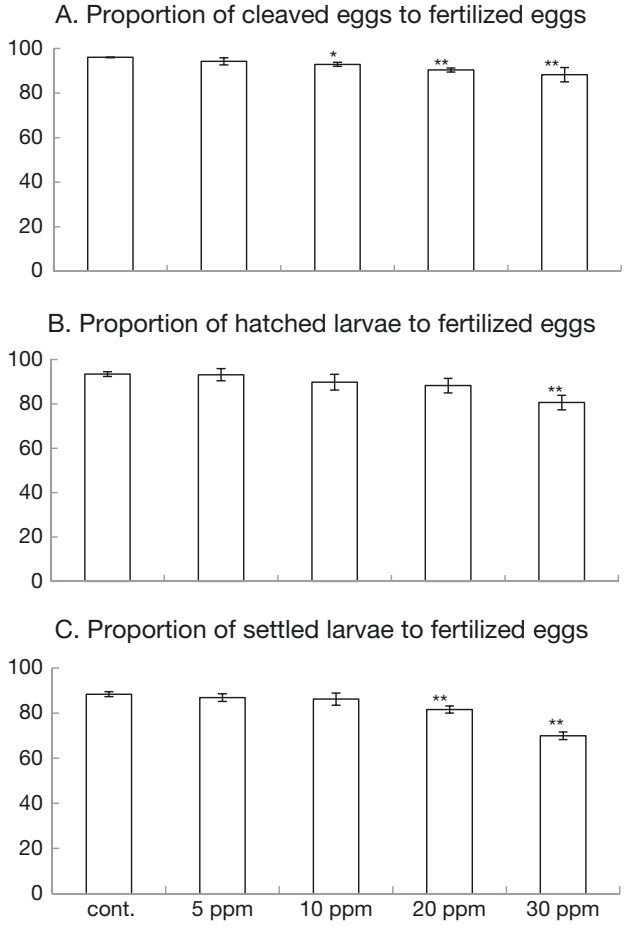

Fig. 1. Effects of disinfection with sodium hypochlorite on the development of fertilized ascidian eggs (mean ± SD). (A) Proportion of cleaved eggs to fertilized eggs; (B) proportion of hatched larvae to fertilized eggs; (C) proportion of settled larvae to fertilized eggs. Asterisks indicate significant differences to control *$p < 0.05$, **$p < 0.01$ (Dunnett's test)

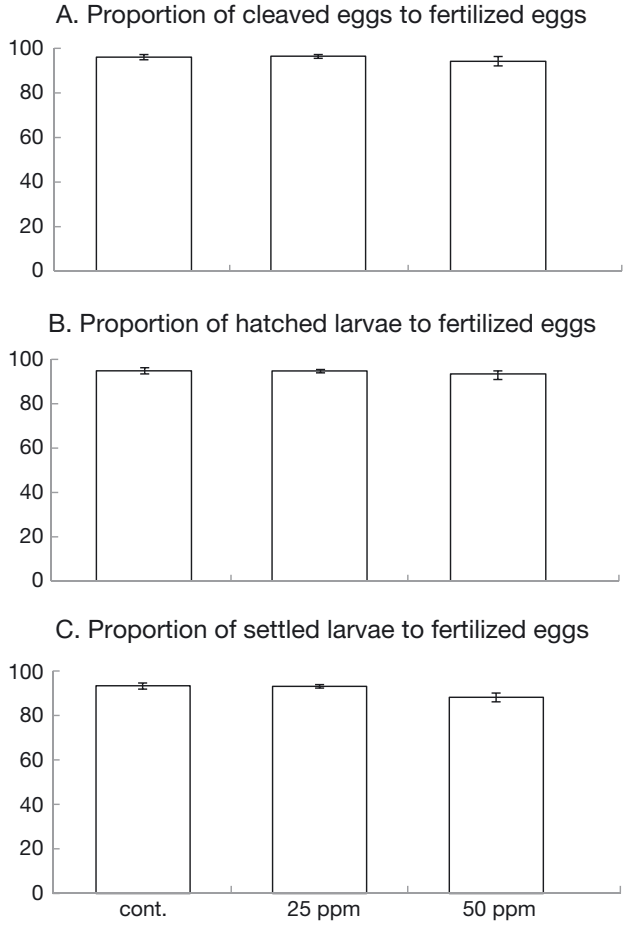

Fig. 2. Effects of disinfection with povidone-iodine on the development of fertilized ascidian eggs (mean ± SD). (A) Proportion of cleaved eggs to fertilized eggs; (B) proportion of hatched larvae to fertilized eggs; (C) proportion of settled larvae to fertilized eggs. No significant difference (Dunnett's test, $p > 0.05$) was found between disinfected groups and the control at any developmental stage

Table 1. *In vitro* disinfectant activity of sodium hypochlorite and povidone-iodine on *Azumiobodo hoyamushi*. Flagellate responses to the disinfection treatments are identified as follows: completely non-motile (–), flagella-only motile (±), abnormal swimming of some flagellates (+), or normal swimming of all flagellates (++)

Concentration (ppm)	Treatment time (min) 0	1	5	10	15
Sodium hypochlorite					
0	++	+	++	++	++
0.25	++	+	++	++	++
0.5	++	+	+	+	+
1	++	–	–	–	–
2	++	–	–	–	–
3	++	–	–	–	–
4	++	–	–	–	–
5	++	–	–	–	–
Povidone-iodine					
0	++	+	++	++	++
1	++	+	++	++	++
2	++	+	++	++	++
3	++	+	++	++	++
4	++	+	±	±	–
5	++	–	–	–	–

temporary cysts and flagellated cells, because they have anatomically similar cytoplasmic contents (Nawata et al. 2015). In the present study, the susceptibilities of *A. hoyamushi* to povidone-iodine solution (5 ppm, 1 min) did not differ between temporary cysts and flagellated cells. The similarities in susceptibility to the disinfectant may be related to the structural similarity between the 2 forms.

The bactericidal activities of povidone-iodine and sodium hypochlorite are reduced by the presence of organic materials (Basha et al. 1998). In the present study, we did not measure the reduction in the available concentration of disinfectants during the treatments. The Japanese salmonid aquaculture industry recommends that up to 5000 eggs can be disinfected with 1 l of povidone-iodine solution (50 ppm) for 15 min. Based on this protocol, we applied the same ratio of fertilized ascidian eggs to volume of povidone-iodine solution in the present study. The diameter of the eggs of ascidians and salmonids are 330 µm and 3–5 mm, respectively. Because ascidian eggs are much smaller and settlement) of ascidian eggs. Additionally, both disinfectants completely inactivated *Azumiobodo hoyamushi* at 2 life stages (flagellate form and temporary cyst form) at a concentration of 5 ppm in 1 min. Therefore, the povidone-iodine solution can be safely used to treat ascidian eggs at up to 10 times the effective concentration for disinfecting *A. hoyamushi*. Conversely, the safe concentration of sodium hypochlorite is equal to the effective concentration. Thus, povidone-iodine could be used more safely than sodium hypochlorite.

Cysts of dinoflagellates are either resting or temporary; the former are able to persist under adverse conditions for a long period, whereas the latter are able to endure short-term or sudden stress but are less resistant to decay than resting cysts (Chapman et al. 1982). Cysts of *A. hoyamushi* are thought to be temporary cysts based on electron microscopic observation of a thin wall and identical cytoplasmic content as the flagellate form (Nawata et al. 2015). There are no differences in temperature tolerance between

Table 2. *In vitro* disinfectant activity of povidone-iodine and sodium hypochlorite against temporary cysts of *Azumiobodo hoyamushi*

Treatment	No. of cysts (cells cm^{-2}) Before disinfection	After disinfection	After incubation	Swimming flagellates
Sodium hypochlorite				
5 ppm, 1 min	777	752	820	No
5 ppm, 5 min	714	720	718	No
Povidone-iodine				
5 ppm, 1 min	846	866	968	No
5 ppm, 5 min	1152	1144	1111	No
Control	7724	734	151	Yes

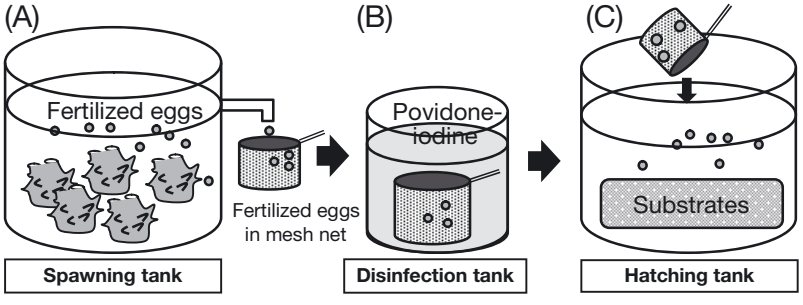

Fig. 3. Summary of ascidian egg-disinfection procedures using povidone-iodine. (A) Collection of fertilized eggs using a mesh net from the spawning tank in which the adult ascidians are reared. (B) Disinfection of the fertilized eggs in the mesh net with povidone-iodine from 5 ppm for 1 min to 50 ppm for 15 min. (C) Placement of the disinfected eggs into a hatching tank. For larval attachment, substrates are put into the hatching tank with seawater filtered through a 0.45 µm filter

than salmonid eggs, any reduction in the available concentration of disinfectants during the treatments would be negligible.

Jang et al. (2012) reported the cyst-like form of *A. hoyamushi* in diseased tunic tissue. Hirose et al. (2014) noted that cysts of *A. hoyamushi* that reside in the substrates on which the spats from Korea attach may be responsible for the outbreaks of soft tunic syndrome in Japan. Additionally, Nawata et al. (2015) revealed that *A. hoyamushi* transforms to a temporary cyst and adheres to both organic and inorganic substrates, and that the temporary cysts can survive for 90 d at 10°C and 95% are capable of excystment. Even if the egg surface of ascidians and the tools used for spawning were contaminated with the flagellate form and/or temporary cyst form of *A. hoyamushi* derived from the carrier of spawners, the disinfection procedures for the eggs and tools using povidone-iodine baths ranging from 5 ppm for 1 min to 50 ppm for 15 min prior to the introduction of eggs into the hatching tank can prevent horizontal transmission of *A. hoyamushi* via the substrates without any side effects on the ascidian development (Fig. 3).

In Korea, mass mortality of cultured ascidians caused by soft tunic syndrome has led to significant economic losses. Hatchery-based seed production of ascidians is common in Korea. Our results suggest that the epizootic area could be contained by disinfecting eggs and tools during hatchery-based seed production and only introducing spats produced under the disinfection procedure into areas where the disease has not yet been detected.

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