用水の紫外線照射によるクドア属粘液胞子虫2種の防除効果

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Short communication

Effectiveness of Ultraviolet Irradiation of Seawater for the Prevention of *Kudoa yasunagai* and *Kudoa amamiensis* (Myxozoa: Multivalvulida) Infections in *Seriola* Fish

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ABSTRACT—*Kudoa* infections have recently become a serious concern in Japanese fisheries. Some species not only cause fish diseases and reduce the commodity value of fish, but also affect human health. We investigated whether the ultraviolet (UV) treatment of culture water prevented infections with two species of *Kudoa*, *K. yasunagai* and *K. amamiensis*, in *Seriola lalandi* and *S. quinqueradiata*, respectively. Rearing in untreated filtered seawater resulted in high infection rates, suggesting that the physical filtration systems used in this study did not sufficiently remove the infective stages of these *Kudoa* species. In contrast, commercially available UV irradiation system effectively prevented the infections with *K. yasunagai* and *K. amamiensis*.

Key words: *Kudoa yasunagai*, *Kudoa amamiensis*, ultraviolet irradiation, prevention

Kudoid myxozoans (Multivalvulida) cause problems in the fisheries industry. Their pathophysiological effects, such as unsightly cyst formations in flesh, spinal deformation, and postmortem myoliquefaction, reduce the commodity value of fish. Moreover, the recent discovery of *Kudoa septempuncata,* which infects the somatic muscle of olive flounder *Paralichthys olivaceus,* as a causative agent of human food poisoning is raising concern over the possible impact of other *Kudoa* species on public health (Kawai *et al.*, 2012). To date, there has been no effective measure of control against *Kudoa* in aquaculture. Although a chemotherapeutic attempt has been made, elimination of myxospores from fish can be extremely difficult (Jones *et al.*, 2012). Therefore, prevention is the most realistic way to control *Kudoa* infection in culture facilities. Cobroft and Battaglene (2012) showed that ultraviolet (UV) irradiation at a dose of > 44 mJ/cm^2 or ozonation of culture water effectively prevented the infection of *Kudoa neurophila* in a striped trumpeter *Latris lineata*. However, whether the same methods are also applicable for other *Kudoa* species remains unknown.

In the present study, we aimed to investigate whether UV irradiation of culture water prevents the infection of *K. yasunagai* and *K. amamiensis* in land-based culture facilities. *K. yasunagai* is a brain-infecting species found in nearly 20 fish hosts around the world (Miller and Adlard, 2012). Infected fish show abnormal behavior and spinal deformation leading to mortality in some cases. At a hatchery in Wakayama Prefecture, Japan, the infection of *K. yasunagai* occurs every year and has caused a high rate of deformation and mortality in hatchery-reared yellowtail amberjack *Seriola lalandi*. *K. amamiensis* is generally considered to be nonpathogenic to fish, but causes unsightly cysts in the somatic muscle and reduces the market value of fish. This species causes problems, particularly in the aquaculture of Japanese amberjack *Seriola quinqueradiata*. In the present study, we reared these two *Seriola* fishes at two different culture facilities in UV-irradiated water and the infection of respective *Kudoa* species was compared with that of fish reared in untreated filtered seawater.

Materials and Methods

Kudoa yasunagai *and* Seriola lalandi

This experiment was conducted during June and July 2013 at a hatchery in Wakayama Prefecture, Japan. Juvenile Japanese amberjack were reared from eggs in water treated with a high dose of UV irradiation (253.7 nm, > 1,000 mJ/cm^2, PO-10M Seabass Ltd.). Thirty-seven-day-old uninfected juveniles were separated into two different size classes (*t*-test; $p < 0.001$), large (mean fork length 6.2 ± 0.48 cm, mean weight 3.1 ± 0.71 g) and small (mean fork length 5.1 ± 0.4 cm, mean weight 1.7 ± 0.4 g). Three hundred fish from each size class were placed into each of two 500-L tanks and reared for 58 days with UV-treated (UV group) or untreated filtered seawater (control group). The control group was exposed to seawater filtered through a fiber-filtration system (Kemari-system, Daiki Ataka Engineering; specified capture particle size ≥ 2 μm, suspended substance removal rate < 95%, linear

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velocity < 50 m/h). For the UV group, the same water was used as in control, but additionally treated with UV (manufacturer's specification; 253.7 nm, 68 mJ/cm^2) (PO-10W, Seabass Ltd.). Water flow was maintained at 200 L/h for all tanks during the course of the experiment and the mean water temperature was 25.2 ± 1.3°C (23.0°C–27.6°C). Rearing density was reduced to 180 fish per tank after 7 wk due to fish growth. The experiment was terminated at 8 weeks as fish grew too large to keep in the experimental tanks.

Starting at 14 days, 10 fish from each tank were examined for *K. yasunagai* infection every wk. Sampled fish were measured and the brain and spinal cord were excised. Collected tissues were homogenized in a 1.5-mL tube and examined for infection using both microscopy and polymerase chain reaction (PCR) analysis. Dissecting equipment was sterilized between each sample by dipping in chlorine solution, to reduce the risk of contamination. For microscopy, a portion of brain/spinal cord homogenate was spread onto a glass slide and stained with Diff-Quik (Sysmex co.) to detect myxospores. For PCR analysis, DNA was extracted from approximately 50 μg of homogenate using a QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol. PCR was performed with primer sets targeted to 28S rDNA of *K. yasunagai,* Kyas f (5′-AGG TTC TCT ACC AAC AAA GTC TG-3′) and Kyas r (5′-CTA GCC CAC TCA AAA TTG CTC-3′). The PCR reaction mixture contained 0.3 μM of each primer, 10 μL of Takara Emerald Amp PCR Master- MIX (2x), and 1 μL template DNA, and was topped up to 20 μL with H_2O. The cycling protocol comprised an initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s, followed by a final extension of 72°C for 10 min. PCR products were separated on a 1.5% agarose gel. Samples diagnosed as positive by either microscopy or PCR were classified as "infected" fish and the infection prevalence was compared between treatment groups using a Fisher's exact test (one-sided test).

Kudoa amamiensis *and* Seriola quinqueradiata

This experiment was conducted during April and October 2013 at a land-based culture facility in Amami Islands, Kagoshima Prefecture, Japan. Ninety hatchery-reared juvenile *S. quinqueradiata* (82 days old, total length approximately 15 cm) were placed in each of two 3,000-L tanks and reared with UV-treated (UV group) or untreated filtered seawater (control) for 6 months. The water for the control group was filtered through a pressure sand filter (YMD-104 A, Nishinihon Crystal Co. Ltd., minimum capture particle size 10–30 μm). For the UV group, the filtered water was treated with UV (manufacturer's specification; 253.7 nm, 216 mJ/cm^2) (PO-10MN, Seabass Ltd.). Water flow was maintained equally for both groups at approximately 3,100 L/h. Fish were maintained at an ambient water temperature and fed every 2–3 days *ad libitum* with commercial dry pellets.

We examined *K. amamiensis* infection in both groups at 4 months (128 days). Since the infection was confirmed, control fish were discarded at this point. The UV group was maintained for another 2 months and re-examined after 6 months (178 days) of the experiment. At each sampling, 20–25 fish were randomly taken from each group and maintained frozen until examination. A half fillet of each individual fish (left side) was taken and sliced at 1 cm width for detecting cysts under a dissecting scope. The cyst of *K. amamiensis* is whitish and round in appearance (approx. 1 mm), and can be easily recognized. When cysts were found, a few were taken from each fish and examined under a light microscope to observe the morphological characteristics of myxospores. PCR was not used for detection of *K. amamiensis* because our previous study showed that microscopy is more reliable to detect low infection than PCR (S. Shirakashi, unpublished data).

Results and Discussion

Kudoa yasunagai *and* Seriola lalandi

Infection was first confirmed after 4 wk in both group sizes by PCR, and the prevalence increased over time, reaching 60% and 90% for large and small groups at 8 wk, respectively. In contrast, *K. yasunagai* was not detected in any of the samples taken from the UV group (Table 1). This indicates that UV treatment with

Table 1. Infection prevalence (%, n = 10) of *Kudoa yasunagai* in two group sizes of *Seriola lalandi* reared in untreated filtered seawater (control) and UV-treated filtered seawater

Fish size	Experimental group	Weeks						
		2	3	4	5	6	7	8
Small	Control	0	0	10	30	60	40 (20)	90 (70)
	UV treated	0	0	0	0	0*	0	0*
Large	Control	0	0	30	10	30	10 (10)	60 (60)
	UV treated	0	0	0	0	0	0	0*

The values in parentheses are prevalence detected by microscopy.
*: significantly different from the control ($p < 0.05$).

a dose of 68 mJ/cm^2 successfully prevented the infection. The infection prevalence in small fish was consistently higher than that of large fish, except on one sampling (Table 1). However, the difference was not statistically significant, due to a smaller sample size. The tendency of a lower infection rate in larger fish may suggest that juvenile *S. lalandi* become more resistant to *K. yasunagai* as they grow. This should be investigated further, but if this is the case, a reduction in the infection rate in open water cages may be made possible by stocking larger juveniles. Myxospores became detectable in the brain after 7 wk. In our previous study we showed that spore formation takes 4 to 8 wk and we expected to find myxospores earlier in the experiment (Shirakashi *et al.*, 2012). This may be partly because we could have overlooked small amounts of spores in the limited amount of brain homogenate. Nevertheless, the basic biology of *K. yasunagai,* including life cycle, route of host invasion, and development within the fish host, remains to be studied. Such information would be useful not only to extend our knowledge about *Kudoa*, but also to develop a new control strategy.

During the experiment, we did not notice any abnormal behavior or deformation, the typical symptoms of *K. yasunagai*. Some mortality occurred mostly at the beginning of the experiment and ceased within a few weeks. Mortalities were similar between groups (a total 22–30 fish/group) and this was probably due to the stress of handling and new experimental conditions. In our previous experiences, the spinal deformation of infected fish becomes noticeable at around 4 to 5 months. The present experiment was terminated when fish were 3 months old, thus the pathological effects of *K. yasunagai* were not observed. We plan to perform a longer experiment to assess the relationship between the development of symptoms, fish size, and infection onset.

K. amamiensis *and* Seriola quinqueradiata

Infection of *K. amamiensis* in the control group reached 100% after exposing fish in untreated filtered seawater for 4 months (Table 2). Cysts had already formed at this point and an average of 11.5 ± 10.4 (1–42) cysts were found per fillet. Cysts were approximately 0.5–1 mm in diameter and visible even with the naked eye and large proportions were observed at the posterior part of the body, particularly along the caudal blood vessel. All observed myxospores possessed four spore valves and showed typical morphological characteristics of *K. amamiensis*. In contrast, no infection was detected in fish from the UV group even after 6 months of exposure in *K. amamiensis*-infested water. This clearly indicates that UV irradiation at a dose of 216 mJ/cm^2 killed or inactivated the infective stage of *K. amamiensis*. The dose used in this study was relatively high due to the availability of equipment, but still within ranges used in hatcheries. Typically, a dose greater than 10 mJ/cm^2 is used for the disinfection of bacteria and some viruses. For micropathogens that are more resistant to UV and for parasites, a dose of over 200 mJ/cm^2 is required (Kasai *et al.*, 2002a, b). It is possible that UV irradiation much lower than 216 mJ/cm^2 may also be useful for preventing *K. amamiensis.* Further experimentation with more frequent sampling is required to determine the minimal effective dose.

Table 2. Infection prevalence (%, number of fish examined in parentheses) of *Kudoa amamiensis* in *Seriola quinqueradiata* reared in untreated filtered seawater (control) and UV-treated filtered seawater

Experimental group	Months	
	4	6
Control	100 (20)	na
UV treated	0* (20)	0 (25)

na: not assessed, *: significantly different from the control ($p < 0.05$).

Conclusions

Our study demonstrates that UV irradiation of culture water is effective in preventing the infection of two species of *Kudoa.* The efficacy of UV irradiation has previously been reported only for *K. neurophila* (Cobroft and Battaglene, 2012). Our results support the idea that infective stages (actinospore) of various *Kudoa* species are probably vulnerable to UV irradiation. However, further investigation is necessary to determine the minimum UV dose required to inactivate the infective stages of *Kudoa* spp. For *K. neurophila*, a UV dose greater than 44 mJ/cm^2 was effective (Cobroft and Battaglene, 2012). We showed that doses of 68 and 216 mJ/cm^2 were effective. However, these are the maximum strength of the UV lights and these values might be reduced over time. Therefore, a lower dose would probably be sufficient to prevent the infection. To date, neither an infective stage nor an invertebrate alternative host for any *Kudoa* species have been found. It may be that the actinospore of *Kudoa* are small and fragile, thus vulnerable to UV irradiation.

Our experiment also showed that fiber-filtration and the pressure sand filter did not completely remove the infective stages of two *Kudoa* species. Similarly, *K. neurophila* infection was not prevented by sand filtration, 50 μm cartridge filter, and foam fractionation (Cobroft and Battaglene, 2012). *K. thyrsites* infection also occurred in an Atlantic salmon *Salmo salar* hatchery using sand-filtered seawater (Moran, 1999). However, prevention of *K. septempunctata* infection using a conventional (gravity) sand filtration system was successful (H. Yokoyama, unpublished data). Physical filtration systems may be effective in some cases, but can

be affected by various conditions such as water flow rate and/or cleanliness of filtration materials and water quality. Therefore, prevention that relies solely on a physical filtration system can be a risky strategy. Filtration is useful for removing particles from the water and thus enhancing the efficacy of UV irradiation. We recommend a combination of physical filtration and UV irradiation for the prevention of *Kudoa*.

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用水の紫外線照射によるクドア属粘液胞子虫２種の防除効果

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近年，クドア属粘液胞子虫による水産業や公衆衛生上の被害が問題となっている。一般に魚体に寄生した粘液胞子虫の駆虫は困難であるため，予防が現実的な対策となる。本研究では，*K. yasunagai* と *K. amamiensis* について飼育水の紫外線（UV）照射による防除を試みた。ヒラマサとブリの稚魚をそれぞれのクドア種が見られる陸上養殖施設で濾過海水（対照区）もしくは市販の装置を用いてUVを照射した濾過海水（UV区）で数ヶ月間飼育した結果，対照区では高率で寄生が見られ，使用した濾過処理では寄生が防げなかった。一方，UV区ではいずれの種も寄生が起らず，UV照射による防除効果が認められた。また，*K. yasunagai* については同一年齢でも小さいヒラマサ稚魚で寄生率が高く，魚体サイズによって感受性に違いがあることが示唆された。

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