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Short Communication

Inter-Laboratory Validation and Applications of Quantitative Real-Time PCR for the Detection of *Kudoa septempunctata* in Olive Flounder (*Paralichthys olivaceus*)

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SUMMARY: *Kudoa septempunctata*, a myxosporean parasite, was recently identified as the causative agent of food poisoning resulting from the consumption of raw olive flounder (*Paralichthys olivaceus*). A single blind inter-laboratory study, involving 5 laboratories, was conducted to validate a quantitative real-time PCR assay for the detection of the parasite. We obtained relatively constant values for log rDNA copies/g from these laboratory analyses (SD = 0.35–0.86), suggesting the validity of the real-time PCR method for the detection of *K. septempunctata* in *P. olivaceus*. Detection of *K. septempunctata* in muscle tissue samples collected from both sides of the fish indicated that *K. septempunctata* infection spreads throughout the body of *P. olivaceus*. *K. septempunctata* infection in *P. olivaceus* is thought to occur during the early stage of fish growth because a *K. septempunctata* gene was detected in 1 of 300 *P. olivaceus* fry tested. Feeds seem not to be sources of infection. To prevent food poisoning due to *K. septempunctata*, the mechanism of infection and proliferation of *K. septempunctata* in *P. olivaceus* should be elucidated, and other hosts of the prasite should be identified. The sensitive real-time PCR method described here will be a useful tool for resolving these issues.

In recent years, the number of cases of unidentified food poisoning manifested as vomiting and diarrhea, 2–12 h after consumption of raw olive flounder (*Paralichthys olivaceus*) in Japan, has dramatically increased to approximately hundreds of cases annually. *Kudoa septempunctata*, a newly identified myxosporean species of Multivalvulida (1), was recently identified as the causative agent of food poisoning associated with the consumption of raw *P. olivaceus* (2). Quantitative detection of *K. septempunctata* from *P. olivaceus* is essential to study this type of food poisoning because symptoms seem to occur by the consumption of a certain amount of *K. septempunctata* (2). We microscopically studied the quantitative distribution of *K. septempunctata* in individual *P. olivaceus* and conducted an inter-laboratory study to validate a quantitative real-time PCR to detect this parasite.

To study the distribution of *K. septempunctata* in individual *P. olivaceus*, we collected muscle tissues samples containing spores of the parasite from various locations on each infected *P. olivaceus* and quantified by microscopically counting them (2). Briefly, each *P. olivaceus* muscle tissue sample was immersed in PBS and filtered through 2 meshes of pore sizes of 200 and 100 μm. The filtrate was centrifuged at 400 $\times$ *g* for 10 min, and the resulting pellet was suspended in the original sample weight of PBS. Spores that have 6 or 7 valves in the suspension were microscopically counted using a hemocytometer. *K. septempunctata* spores were detected from all muscle tissue samples examined, except one, collected from 10 different locations (5 locations each on the side with eyes and without eyes) on each of 4 *P. olivaceus* (Table 1). In a *P. olivaceus* (fish D) sample, $<1 \times 10^4$ spores/g of tissue were identified, which was less than the microscopic detection limit. We found that the standard deviation increased with a decrease in the number of *K. septempunctata* spores. This finding suggests that *K. septempunctata* infection spreads throughout the body of *P. olivaceus* to varying extents, although the mode of infection and spread of *K. septempunctata* is unclear.

A quantitative real-time PCR method was designed to detect *K. septempunctata*. A single blind inter-laboratory study, involving 5 laboratories, was conducted to validate the results of the quantitative real-time PCR assay. In all, 40 test samples from 20 *P. olivaceus* (2 samples of muscle tissues from each *P. olivaceus*) known or suspected to have caused cases of food poisoning were distributed to each laboratory. *K. septempunctata* DNA was extracted from the muscle tissue samples of *P. olivaceus* using a QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's tissue protocol instructions, and it was analyzed at all the laboratories by the real-time PCR method. The following primers and probe were used: Kudoa-F (5′-CATGGGATTAGCCCGGTTTA-3′), Kudoa-R (5′-ACTCTCCCCAAAGCCGAAA-3′), and Kudoa-P (5′-FAM-TCCAGGTTGGGCCCTCAGTGAAAA-TAMRA-3′). The thermal cycling conditions of the real-time

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Table 1. Distribution of *K. septempunctata* spores in individual *P. olivaceus*

ID of spot	*K. septempunctata* in *P. olivaceus* (log spores/g)			
	ID of *P. olivaceus*			
	A	B	C	D
1	6.95	6.48	6.53	<4[1)]
2	6.74	6.46	6.49	5.62
3	6.76	6.48	6.79	5.73
4	6.95	6.43	5.79	5.88
5	6.74	6.15	6.86	5.61
6	6.84	6.38	5.48	5.00
7	6.62	6.25	5.81	5.94
8	6.90	6.08	6.34	6.00
9	6.55	6.18	6.13	6.18
10	6.83	6.29	6.21	5.82
Mean	6.79	6.32	6.24	5.58
SD	0.13	0.14	0.43	0.61

1): Less than detection limit (4 log spores/g). Value was set at 4 for purposes of calculating the mean and standard deviation.

PCR were as follows: 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 72°C. Plasmid DNA containing *K. septempunctata* 18S rDNA was used as a standard. *K. septempunctata* spores in the same samples were microscopically counted at the National Institute of Health Sciences. The spores were detected in 10 of the 20 *P. olivaceus* examined, but the spores were less than the microscopic detection limit in the other 10 *P. olivaceus*. The log rDNA copies/g of tissue values obtained from the different laboratories were relatively constant (SD = 0.35–0.86) (Table 2), though the *P. olivaceus* tested in the present study might have been infected with *K. septempunctata* to varying extents (Table 1). These results suggest that real-time PCR is a valid method for detecting *K. septempunctata* in *P. olivaceus*, and it is capable of identifying the organism at the lower end of the microscopic detection limit. The results of this inter-laboratory study suggested that the real-time PCR system was sensitive, but not absolutely specific. The method showed cross reactivity with some other *Kudoa* spp., including *K. thyrsites* and *K. lateolabracis*.

We applied the real-time PCR assay and a microscopic method to study the prevalence of *K. septempunctata* spores in the remnants of *P. olivaceus* that caused food poisoning, *P. olivaceus* fry, and feed materials used in *P. olivaceus* aquaculture. In all, 61 *P. olivaceus* remnants known or suspected to cause food poisoning were analyzed for the presence of *K. septempunctata* spores. We could not detect spores in 17 of 61 samples by microscopic analysis; however, *K. septempunctata* genes were detected in these 17 samples by the real-time PCR assay. Both *K. septempunctata* spores and genes were detected in the remaining 44 samples. A plot of the number of spores versus the 18S rDNA copy number is shown in Fig. 1. The 18S rDNA copy number moderately correlated with the number of spores (r^2 = 0.70). *P. olivaceus* carrying 6–7 log spores/g were the most prevalent (n = 27), followed by fish carrying 5–6 log spores/g (n = 8) and 7–8 log spores/g (n = 6). None of the *P. olivaceus* examined carried more than 8 log spores/g. The 17 samples in which no spores were detected were found to contain 1.44–4.85 log copies of rDNA/g (mean, 3.35 log copies of rDNA/g) by the microscopic analysis.

We also determined the copy number of 18S rDNA in each *K. septempunctata* spore using purified samples obtained by Percoll gradient centrifugation (2). Unfor-

Table 2. Presence of *K. septempunctata* DNA and spores in various samples of olive flounder (*P. olivaceus*)

ID of *P. olivaceus*	*K. septempunctata*	
	log rDNA copies/g	log spores/g
1	10.54 ± 0.35[1)]	7.65
2	9.35 ± 0.37	7.88
3	9.67 ± 0.41	7.02
4	9.35 ± 0.39	6.67
5	9.49 ± 0.42	7.20
6	9.13 ± 0.85	6.83
7	9.47 ± 0.41	6.71
8	9.11 ± 0.42	6.57
9	8.87 ± 0.59	6.34
10	6.55 ± 0.40	4.85
11	5.76 ± 0.45	<4[2)]
12	6.79 ± 0.47	<4
13	6.93 ± 0.23	<4
14	6.23 ± 0.38	<4
15	6.46 ± 0.57	<4
16	6.62 ± 0.48	<4
17	6.65 ± 0.35	<4
18	5.01 ± 0.72	<4
19	4.84 ± 0.86	<4
20	5.56 ± 0.57	<4

1): Means and standard deviations of 10 test samples analyzed at 5 different laboratories.
2): The number of spores was less than the microscopic detection limit.

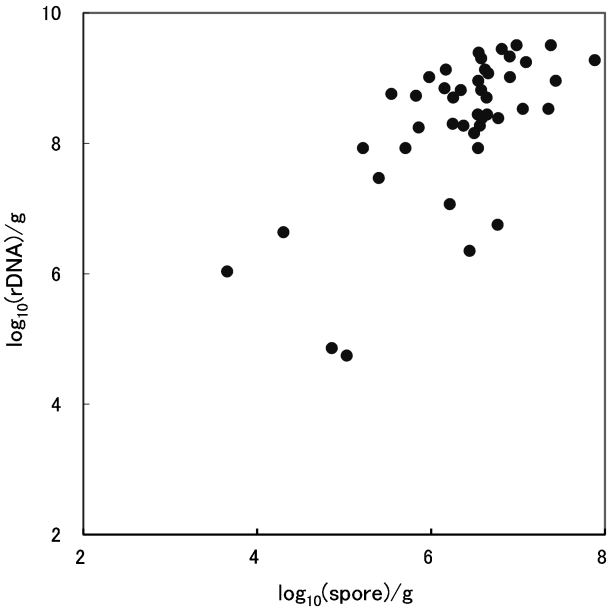

Fig. 1. Scatter plot of *K. septempunctata* spore number and 18S rDNA copy number in 44 *P. olivaceus* samples.

tunately, the results were not reproducible, but we found that a spore might contain 2–3 log rDNA copies. The copy number/spore data were also supported by the findings of the analyses of the 10 samples, shown in Table 2 and the 44 samples, shown in Fig. 1. At present, the importance of the spore count or rDNA copy number is unknown because the pathogenicity of *K. septempunctata* is obscure. Analyses of both spore count and rDNA copy number would contribute to clarify the mechanism of food poisoning.

The life cycles of myxosporean parasites, including the genus *Kudoa*, are poorly understood and found to be complex. Several species of the myxosporean have been reported to have complex life cycles that involve a myxospore stage in fish and an actionospore stage in annelid worm (3–5). The life cycle of *K. septempunctata* is not known, with an exception of the formation of myxospore, which has 6 or 7 valves in *P. olivaceus* (1). Further, *K. septempunctata* is suspected to have a presporogonic stage at which the microscopic detection of the myxospores is not possible. In the present study, the spore number was found to be moderately correlated with the 18S rDNA copy number, and this may be attributed to the negligible detection at the presporogonic stage of *K. septempunctata* in *P. olivaceus*.

Myxosporean parasites, including those belonging to the genus *Kudoa*, may infect fish via various routes such as through the digestive tract, gills, skin, and fins; however, the mechanism of infection remains to be elucidated (4,6). To evaluate whether feed can also be a source of infection, we analyzed 4 types of feed material (3 lots of rotifer, 3 lots of brine shrimp, 4 lots of granular assorted feed A, and 1 lot of granular assorted feed B) by both real-time and conventional PCR assays. Briefly, 10 g of the feed was mixed with 90 ml of PBS and homogenized with Waring blenders (Nihonseiki, Tokyo, Japan). DNA was extracted using a QIAamp DNA Mini Kit and then analyzed by PCR. The thermal cycling conditions of the conventional PCR were as follows: 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. The following *Kudoa*-specific primers were used: SSU1 (5′-ACCAA GGTTGTGACGGGTAA-3′) and SSU3 (5′-AGGCTC AGTCCAAAGCAAGA-3′) (7). The assorted feed B consisted of rDNA gene with 99.9% sequence identity with the *Kudoa* sp. MMAG-5, whereas the other feeds contained no genes derived from *Kudoa* spp., indicating that feed is not a source of infection.

We also analyzed *P. olivaceus* fry for *K. septempunctata* genes. In all, 300 fry were obtained from a domestic fry supplier (total length, 40–60 mm) and analyzed by the real-time PCR method. The 18S rDNA of *K. septempunctata* was detected in a single fry, suggesting that *K. septempunctata* infection of *P. olivaceus* occurs during the early stages of current fish farming.

Details regarding the pathogenicity of *K. septempunctata* remain to be elucidated. Its pathogenicity in humans; the mode, specificity of infection, and proliferation in *P. olivaceus*; and infection in other hosts are unknown. Further studies are required to better understand these details in order to prevent food poisoning caused by this parasite. The sensitive real-time PCR method described here would be a useful tool for resolving these issues.

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Conflict of interest None to declare.

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