Detection and Identification of *Miamiensis avidus* Causing Scuticociliatosis by PCR

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ABSTRACT—We designed a PCR primer set for specific detection of *Miamiensis avidus* causing scuticociliatosis in Japanese flounder *Paralichthys olivaceus*. In the PCR targeting the small subunit ribosomal RNA gene of *M. avidus*, the expected PCR product with 1,433 bp was amplified from four isolates of *M. avidus* including three different serotypes, but not from other scuticociliates including *Pseudocohnilembus persalinus*, *P. hargisi* and *Uronema marinum*. Detection limit of the present PCR was 125 fg of genomic DNA. When a group of Japanese flounder suffering the disease was examined by the PCR, 90% of symptomatic fish and 50% of asymptomatic fish were found positive. Thus it is considered that the PCR is useful for detection and identification of *M. avidus* and consequently for diagnosis of scuticociliatosis in Japanese flounder.

Key words: scuticociliatosis, *Miamiensis avidus*, PCR, *Paralichthys olivaceus*, detection

Scuticociliatosis is one of the most important parasitic diseases in cultured Japanese flounder *Paralichthys olivaceus* in Asian countries such as Japan, Korea and China. This disease is characterized by signs with severe ulcer and haemorrhage in skeletal muscle, moreover scuticociliates often invade fish systemically. However, there are fish without these external signs, in which the affected tissue is limited to a brain. The causative agents of scuticociliatosis include *Uronema marinum*¹⁾, *Philasterides dicentrarchi*²⁾, *Pseudocohnilembus persalinus*³⁾, *Ps. hargisi*³⁾ and *Miamiensis avidus*^{4,5)}, but it has been suggested that *M. avidus* and *Ph. dicentrarchi* are synony-

mous based on morphological and genetic analyses^{5,6)}. In the challenge experiments to Japanese flounder using *M. avidus*, *Ps. persalinus*, *Ps. hargisi* and *U. marinum*, high mortalities were only observed in the fish challenged with *M. avidus*, suggesting that the ciliate is etiologically important in the endoparasitic scuticociliatosis of Japanese flounder⁷⁾. Moreover, it was revealed that at least three serotypes existed in Japanese isolates of *M. avidus* based on western blotting profile and immobilization assay using antisera against four *M. avidus* isolates; serotype I represented by Iyo I, serotype II by Nakajima and serotype III by Mie0301⁸⁾.

Currently, there has been no chemical treatment to cure endoparasitic scuticociliates. Some effective vaccines for scuticociliatosis have been developed^{9,10)}, but not commercialized in aquaculture markets. It is thus considered that rapid, sensitive and specific detection of *M. avidus* from fish at initial infection phase is important and useful to reduce economic damage. However, other scuticociliates, *Ps. persalinus* and *Ps. hargisi*, existing on fish skin could make confuse detection of *M. avidus*. Thus, in the present study, a specific, sensitive and rapid PCR method for detection of *M. avidus* is newly designated and evaluated.

Materials and Methods

Scuticociliates

As *M. avidus*, a Korean isolate, YS1⁴⁾, and three Japanese isolates, Iyo I (serotype I), Nakajima (serotype II) and Mie0301 (serotype III)⁸⁾, were employed in the present study. *Ps. persalinus* (SCL-A)⁷⁾ and *Ps. hargisi* (SCL-B)⁷⁾ were provided by Dr. S. J. Jung at Chonnam National University, Korea, and *U. marinum* (WSW) was isolated from rearing seawater at the Fish Culture Center of Tottori Prefecture, Japan. These isolates were maintained in 10 mL YEHS broth (2% Lab-Lemco powder (Oxoid), 0.5% Bacto™ yeast extract (Difco), 0.5% glucose, 0.8% sodium chloride and 5% inactivated horse serum; Dr. T. Nakatsugawa, personal communication).

Specific PCR for detection of *M. avidus* (M.a.-PCR)

For designing a PCR primer set, small subunit ribosomal RNA (SSU rRNA) gene was targeted, because the target nucleotide (nt) sequences of Korean and Japanese *M. avidus* were identical to each other^{4,8)}. The target gene sequence of *M. avidus* (GenBank accession number, AY550080) was compared with those of other three scuticociliates, *Ps. persalinus* (AY551906), *Ps. hargisi* (AY212806) and *U. marinum* (Z22881). An *M. avidus*-specific primer set, Ma-F (5'-GTA ACT GAT CGA ATC TCT TCA C-3') and Ma-R (5'-TTC CCG TTC ACG CAA GCG T-3'), was designed at 252 to 1,684 base in the SSU rRNA gene of *M. avidus*. The nt sequence of the forward primer, Ma-F (22 mer),

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mismatched at 5 nt with that of *Ps. persalinus*, 5 nt with that of *Ps. hargisi* and 7 nt with that of *U. marinum*, while nt sequence of the reverse primer, Ma-R (19 mer), mismatched at 10 nt with that of *Ps. persalinus*, 12 nt with that of *Ps. hargisi* and 12 nt with that of *U. marinum*.

Approximately 10^5 of scuticociliate cells were collected by centrifugation at $25,000 \times g$ for 5 min at 4°C from 1 mL of culture fluid, and DNA templates were extracted using a DNeasy Blood & Tissue kit according to the manufacturer's instructions (Qiagen).

PCR was performed in 20 μL of reaction mixture containing 50–100 ng of genomic DNA, 10 μM of each primer, 0.5 U of Ex *Taq* hot start version (TaKaRa), 2 μL of 10 $\times$ Ex *Taq* buffer and 0.2 mM dNTPs. The cycle conditions were 1 cycle at 95°C for 5 min, 40 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 1.5 min, followed by 72°C for 5 min. PCR products were analyzed by 2% agarose gel electrophoresis.

Detection limit of *M.a.*-PCR

To assess the detection limit of this PCR, the genomic DNA was extracted from YS1 isolate by the method described above. An 125 ng/ μL of the genomic DNA was serially diluted ten-fold with distilled water and subjected to *M.a.*-PCR.

Detection of *M. avidus* from affected fish

Detection of *M. avidus* was performed using Japanese flounder surviving from scuticociliatosis occurred at the Fish Culture Center of Tottori Prefecture, Japan in 2009. Each ten fish with and without scuticociliatosis signs (means body weight: 11.5 g) were collected from the same rearing tank. Abrasion and/or ulcer in the fin, muscle, mouth or outside of the gills were observed in these symptomatic fish. Brain, dorsal muscle of ocular side and gills were obtained, while epidermal mucus was collected from the part without abrasion and ulcer by a sterilized spatula. Total DNA was extracted from 50 mg of these samples using a DNeasy Blood & Tissue Kit, and subjected to the PCR.

Results and Discussion

PCR products with the expected size (1,433 bp) were amplified from genomes of all *M. avidus* isolates including three different serotypes, but not from genome of *Ps. persalinus*, *Ps. hargisi* or *U. marinum*. In our preliminary study, no difference was observed in the nucleotide sequences of SSU rRNA among Japanese and Korean *M. avidus* isolates including three serotypes (data not shown). Moreover, nucleotide sequences of the designed primers, Ma-F and Ma-R, showed high E value (> 0.32) to those of other parasitic SSU rRNA genes deposited in DNA database (data not shown). It is considered that *M.a.*-PCR is highly spe-

Fig. 1. Specific detection of *Miamiensis avidus* by *M.a.*-PCR. Lane M : DNA size marker, Lane 1: *M. avidus* (YS1), Lane 2: *M. avidus* (Iyo I), Lane 3: *M. avidus* (Nakajima), Lane 4: *M. avidus* (Mie0301), Lane 5: *Ps. persalinus* (SCL-A), Lane 6: *Ps. hargisi* (SCL-B), Lane 7: *U. marinum* (WSW), Lane N: negative control (distilled water).

Fig. 2. Detection limit of *M.a.*-PCR. Lane M: DNA size marker, Lane 1: 125 ng, Lane 2: 12.5 ng, Lane 3: 1.25 ng, Lane 4: 125 pg, Lane 5: 12.5 pg, Lane 6: 1.25 pg, Lane 7: 125 fg, Lane 8: 12.5 fg, N: negative control (distilled water).

cific to *M. avidus*.

The detection limit of the *M.a.*-PCR was 125 fg of genomic DNA (Fig. 2). It is difficult to calculate that 125 fg of the genomic DNA correspond to how many cells' genomic DNA because recovery rate of genomic DNA is not clear. However, the obtained genomic DNA was extracted from approximately 10^5 cells, meaning that detection limit of the preset *M.a.*-PCR seems to be less than a few cells of *M. avidus*.

Results of detection of *M. avidus* by *M.a.*-PCR from symptomatic and asymptomatic fish were shown in Table 1. In the symptomatic fish, a total detection rate of *M. avidus* were 90%, and those from brains, muscles, gills and epidermal mucus were 80%, 50%, 30% and 10%, respectively. These results demonstrated that detection rate of *M. avidus* in brain was higher than those from other tissues. It was reported that numerous scuticociliates were observed in central nervous system including meninges, telecephalon, diencephalon, optic lobes, cerebellum and medulla oblongata by histological examinations^{5,11)}. Paramá *et al.*¹²⁾ detected scuticociliates in brain tissue within 15 days after experimental infections. It is thus considered that brain is the most suitable tissue for detection of *M. avidus*. Interestingly, detection rate of *M. avidus* from the epidermal mucus was lower than that of other tissues even though abrasion and/or ulcer were observed in body surfaces of the affected fish. This suggests that the epidermal mucus contains some inhibitors for PCR amplification, meaning that modification of the DNA extraction procedure from mucus seemed to be required in the future study. In one symptomatic fish exhibiting ulcer around mouth, *M. avidus* was not detected from any tissues (Table 1). This was hard to understand for us, but we considered that the observed ulcer could be caused by

Table 1. Detection of *M. avidus* from Japanese flounder by *M.a*-PCR

Fish group	No	Organs				total
		Brain	Muscle	Gills	Epidermal skin mucus	
Symptomatic fish (N=10)	1	+	+	+		+
	2	+				+
	3	+	+	+		+
	4		+			+
	5	+	+			+
	6	+				+
	7					
	8	+				+
	9	+	+	+	+	+
	10	+				+
	Detection rate	80%	50%	30%	10%	90%
Asymptomatic fish (N=10)	1	+				+
	2	+	+	+		+
	3	+				+
	4		+	+		+
	5					
	6					
	7					
	8	+				+
	9					
	10					
	Detection rate	40%	20%	20%	0%	50%

physical damage such as friction, not by *M. avidus* infection.

In the asymptomatic fish, a total detection rate of *M. avidus* was 50%, and those from brains, muscles, gills and epidermal mucus were 40%, 20%, 20% and 0%, respectively (Table 1). It was demonstrated that the present PCR is possible to detect *M. avidus* also from asymptomatic survivor fish. Furthermore, the detection rate from brain was higher than those from other tissues. It is thus confirmed again that brain is the most suitable tissue to detect *M. avidus* by the present PCR. From these results, it is concluded that the present PCR could be useful tool for specific detection and identification of *M. avidus*, and also for diagnosis of scuticociliatosis in Japanese flounder.

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