Experimental Scuticociliatosis in Japanese Flounder (Paralichthys olivaceus) Infected with Miamiensis avidus: Pathological Study on the Possible Neural Routes of Invasion and Dissemination of the Scuticociliate inside the Fish Body

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ABSTRACT. Japanese flounder (Paralichthys olivaceus) were experimentally infected with the highly pathogenic scuticociliate Miamiensis avidus (syn. Philasterides dicentrarachi) using the immersion method to clarify/identify the possible neural routes of entry and possible ways of dissemination of the scuticociliate in the fish body. Scuticociliates were observed on the skin and gills right from day 0–1 post-infection, muscle tissue on day 2 post-infection, reached the brain, and spinal cord on day 3 post-infection, and systemic infection was prominent afterwards. Brain lesions were observed in most of the examined fish from days 3 and 4 post-infection and considered to be the cause of the sudden increase in mortality. Affected fish showed varying degrees of tissue damage including severe epidermal and dermal necrotic lesions, necrotic myositis, encephalitis and myelitis. Whereas, scuticociliates were frequently observed along the optic and/or olfactory nerve in the fish which were accompanied by severe brain lesions but by minimum lesions in the gills and skin, suggesting that in addition to skin and/or gills, neural routes including periorbital and nasal routes may play a role in scuticociliate invasion to the brain. Scuticociliates were also observed in the peripheral nerve fibers in the muscle tissue, cranial and spinal nerves, cranial cavity and in the vertebral canal, suggesting that nerve fibers and/or cerebrospinal fluid circulation may be involved in the spread of the scuticociliate throughout the body in addition to the blood circulation and connective tissue.

KEY WORDS: experimental infection, Japanese flounder, Miamiensis avidus, neural routes, scuticociliatosis.

Scuticociliates are free-living organisms in sea-water, feeding on suspended particulate matters (bacteria, microalgae, protozoa). Under certain circumstances, however, these ciliates may behave as opportunistic histophagous parasites, and actively feed on cells and tissue residues of certain mollusks, crustaceans and fishes, and continue to live and reproduce within the host tissues [5]. Several scuticociliate species have been reported as agents to cause scuticociliatosis in farmed marine fish; species of Philasterides dicentrarachi infects sea-bass [3] and population of sea-ium fishes [2].

In Korea, scuticociliatosis in cultured Japanese flounder (Paralichthys olivaceus) was reported to be due to Uronema marinum [8], Philasterides dicentrarachi [12], Pseudocohnilembus persalinus [13] and Miamiensis avidus [10]. It was suggested that Miamiensis avidus is the main cause of scuticociliatosis in Japanese flounder in a series of reports [10, 11, 20].

Recently, a successful experimental immersion infection of Japanese flounder (Paralichthys olivaceus) by Philasterides dicentrarachi was performed in Korea. It was suggested that P. dicentrarachi is a strong pathogen that can cause a primary infection in flounder by penetrating the gills and skin, followed by its travel via the blood stream to the other parts of the body [9]. Jung et al. [11] demonstrated that M. avidus successfully invades the host directly from seawater, and causes high mortality. The ciliates rapidly invade and proliferate in the skin and gills, and spread to the internal organs in the absence of any additional pathogens such as secondary bacterial invaders. These reports demonstrated abraded skin and/or gills as the main routes of entry and blood vessels as the dissemination route [9, 11]. Based on the results from the experimental infection study by intracoelomic injection of the ciliates to the turbot, Puig et al. [17] suggested the connective tissue as a possible quick way of dissemination of the scuticociliate to the body.

Munday et al. [15] proposed that the scuticociliate Uronema nigricans entered its host blue fin tuna by the nasal route; the starting point of infection was traced to the olfactory rosettes, from which the parasite moved up the olfactory nerve to the brain, leading to locomotor dysfunction and final death. Experimental infections by Philasterides dicentrarachi using different routes were successfully attempted in turbot by Parama et al. [16]; after periorbital inoculation of P. dicentrarachi, all fish died within 5–13 days post-inoculation and the infection lesions were largely localized in brain (100% of the fish). Brain infection was observed in all the fish together with the pres-

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ence of ciliates in the optic nerve microscopically, suggesting that *P. dicentrarchi* is able to reach the brain not only via the blood stream but also via nervous tissues [16].

In Japan, though the cause of the outbreak of scuticociliatosis in Japanese flounder (*Paralichthys olivaceus*) was considered to be unidentified ciliate [23] for many years, it has recently become clear that the main cause of the scuticociliatosis is *Miamiensis avidus* as demonstrated in our previous study [6]. Exophthalmia, protrusion of eye ball, was detected in many affected fish accompanied by brain lesions; the findings enabled us to focus on the significance of the neural route for entry and dissemination of the scuticociliate.

The aim of this study is to clarify/identify the possible neural routes of entry and possible ways of dissemination of the ciliates in the fish body through the pathological study of Japanese flounder (*Paralichthys olivaceus*) experimentally infected by immersion method with a scuticociliate, *Miamiensis avidus*.

**MATERIALS AND METHODS**

*Ciliates isolation and cultivation*: Scuticociliates were isolated from the brain of naturally infected Japanese flounders reared at Tottori Prefectural Fisheries Experimental Station in Japan during an outbreak of the disease in 2005. A small piece of the brain of Japanese flounder containing active ciliates was inoculated into *Epithelioma Papillosum Cyprini* (EPC) cells previously cultured in MEM3 [Minimum Essential Medium (Invitrogen, Tokyo, Japan) supplemented with 3% fetal bovine serum and 1% Antibiotic-Antimycotic, liquid (Invitrogen)]. Scuticociliates multiplied with EPC were then kept at 25°C for a period of overnight to 3 days. For subculture, 1 µl of the culture supernatant including the scuticociliates was added to 10 ml of YEHS [Yeast Extract Horse Serum liquid medium: 2% ‘Lab-lemco’ powder (Oxoid, Tokyo, Japan), 0.5% Bacto™ Yeast Extract (Difco 0.5% glucose, 0.8% sodium chloride and 5% inactivated horse serum)], and incubated at 18°C. The scuticociliates were sub-cultured and maintained in the medium, and the concentration of the scuticociliate in the culture medium was approximately 107 cells/ml.

The isolates were morphologically and genetically identified as *Miamiensis avidus*, and classified serologically, using immobilization assays and Western blotting, into “serotype I” and it was named JF05To [21].

**Aquarium**: In the present study, 11 acrylic tanks of 30 l capacity containing seawater, filtered with cartridge filter (0.5 µm, Millipore, Tokyo, Japan), were employed. Each of these tanks contained one perforation at the upper part of a side wall for water drainage. A net was attached to the perforation in the tank to prevent the outflow of the young fish.

**Fish**: All fish used in the experiment (143 young fry of Japanese flounder of total length: 27.7 mm) were reared until the 94th day after hatching in the institution controlled under the same conditions as those in the laboratory in Tottori Prefectural Fisheries Experimental Station in Japan. Compound fish feeds were fed to the fish fries. Two to three days before initiation of the experiment, fish were stopped feeding. Fifteen randomly selected fish were examined under a stereo microscope before necropsy to ensure that they were free from the parasites in the brain, gills, muscle and epidermal skin mucus. Fish were divided into 11 experimental groups and each group was reared in a separate tank. The first 10 groups contained 13 (Tank 1), 10 (2), 10 (3), 10 (4), 10 (5), 15 (6), 15 (7), 15 (8), 15 (9) and 20 (10) fish, respectively and the 11th group contained 10 fish as the negative control. The fish in each tank were sampled in the manner to ensure the minimum number for the investigation and to avoid an intentional selection of weakened individuals. Because of the predicted increased risk of mortality of the fish associated with progression of the disease, the initial numbers of fish in latter tanks were increased so that the total number of the fish examined in each tank would be equal (Miwa and Nakayasu [14]).

All tanks were placed in a big water bath provided with a titanium heater to maintain a steady temperature of 20°C. Water in the fish tanks was slightly aerated constantly during the experiment.

**Experimental infection**: On the first day of infection experiment, each tank was filled with 10 l of filtered seawater and fish were released into the tanks in numbers stated above.

Ten milliliters of the culture medium holding 106 cells of the scuticociliate was centrifuged at 3,500 rpm for 10 min at room temperature. For preparation of concentrate of the ciliates, the supernatant was discarded and the sediment was suspended with 1 ml of filtered seawater. Ten equal concentrates of the scuticociliates were prepared for immersion. These concentrates were added to each of tanks 1 to 10. The concentration of the ciliate in the immersion tanks was approximately 100 cells/ml. At 24 hr later, a further 20 l of filtered seawater were added to each tank. From the next day onwards, half of the water in each tank was removed and replaced by 15 l of filtered seawater on a daily basis. The fish were observed for maximally ten days after the infection without feeding.

Just 30 min after the start of immersion (infection), five live fish were sampled from tank 1. From the first day to the tenth day after the infection, five live fish were sampled from each of tanks 1 to 10 on each day and examined for the possible neural routes of entry and ways of dissemination of the ciliates (Table 1). The fish were checked from the surface to the interior of the body using stereo microscope before necropsy in order to examine the evidence of infection. Fish were euthanized with an exposure of eugenol [Fish/Crustacea anesthetic FA100 (Tanabe Seiyaku Co., Ltd., Osaka, Japan)]. All dead fish of each tank were sampled upon confirmation of the death without consideration about the duration after the infection. All the fish, live and dead, were fixed in Davidson’s solution (330 ml of 95% ethanol, 220 ml commercial formaldehyde solution containing 35% formaldehyde and 8% methanol, 115 ml glacial acetic acid and 335 ml distilled water). After one week, David-
son’s solution was switched to 75% ethanol solution.

**Histopathology:** After gross examination, all fish stored finally in 75% ethanol were transported to the laboratory of Veterinary Pathology of Tottori University, cut into transverse sections, dehydrated and embedded in paraffin wax. Subsequently, sections 2–5 μm thick were stained with hematoxylin & eosin (HE) for light microscopy.

**RESULTS**

**Clinical symptoms:** Bleached spots on the skin and dermal necrotic lesions were the first observable clinical symptoms, which appeared just 30 min after the infection. Scuticociliates were observed on the skin and fins from day 1 post-infection, and detected to spread to the organs as follows; gills, eyes, brain and spinal cord from day 3 post-infection; nostrils from day 4 post-infection; lips and mouth from day 8 post-infection (data not shown). Few fish in the control group were found dead during the course of the study, with detecting the infection of ciliates in some of them. However, PCR examination was negative for *Miamiensis avidus* (data not shown).

**Scuticociliates dissemination inside the body:** Results of the histological detection of *Miamiensis avidus* organism in the experimentally infected fish are summarized in Table 1. The first ciliate infection was observed in the skin and gills in individuals of the group sampled on day 0 (30 min after the immersion). High densities of scuticociliates were observed in the epidermis and gill mucosal epithelium. On day 1 post-infection, the number of fish with evidence of infection and scuticociliates densities within the affected areas remained unchanged. There was an increase in severity of the lesions as demonstrated by the extensive epidermal damage associated with epidermal ulcer. Then, the scuticociliates gradually disseminated within the entire body and appeared in subcutaneous connective tissue, muscle tissue and peripheral nerve endings on day 2 post-infection, in the periorbital cavity surrounding the eye, eye, optic nerve, brain, spinal nerve and spinal cord on day 3 post-infection, in the nasal cavity disseminating into the olfactory nerve towards the brain from day 4 post-infection and in the gastrointestinal tract, liver and kidney from days 4 and 5 post-infection onwards. Since then, the infection was generalized and systemic; brain infection was observed in most of the examined fish. The scuticociliates distribution became more evident by both ciliate density and numbers of the lesions in the affected organs and tissues.

**Histopathology:** Histopathological examination on day 1 post-infection revealed extensive epidermal destruction, subsequent dermal degeneration with lymphocytic, monocytic and eosinophilic granular cell infiltration associated with the presence of a high number of the scuticociliates. On day 2 post-infection, there was necrotic degeneration in the muscles together with the scuticociliates invading underneath the epidermal layer and in the subcutaneous connective tissue. The scuticociliates were also observed in the
nerve bundles (Fig. 1A) and nerve fiber endings (Fig. 1B) in the muscle tissues affected. On day 3 post-infection, severe infection of the scuticociliates was observed in the periorbital cavity (Fig. 2A), eye, optic nerve accompanied by inflammatory cells infiltration (Fig. 2B). The scuticociliates were also observed in the brain, spinal cord, spinal nerve (Fig. 3) and in the vertebral canal (Fig. 4). From day 4 onwards, the scuticociliates were observed in the blood vessels of a variety of tissues and/or organs (Fig. 5), nasal cavity, along the olfactory nerve and in the olfactory lobe, where severe inflammatory changes were detected (Fig. 6A and 6B) and third ventricle. Since then, the infection became more systemic and scuticociliates were observed in the lamina propria and lamina muscularis of the gastrointestinal tract and in the abdominal cavity. Periglomerular, peritubular and perivascular mononuclear inflammatory cells infiltration along with the presence of the scuticociliates inside blood vessels were observed in the kidney. From day 6 onwards, many scuticociliates containing red blood cells in the cytoplasm were observed in the gills, skeletal muscles, skin and brains of the fish.

DISCUSSION

*Miamiensis avidus* is a highly invasive and destructive histophagous endoparasitic scuticociliate infecting Japanese flounder. In the present study, an experimental infection of Japanese flounder was successfully achieved by immersion method to clarify/identify the possible neural routes of entry and ways of dissemination of the scuticociliate in the fish body.

Although there was a slight difference in the density of the scuticociliates between the five alive fish samples from each tank which may give an impact on the course of the pathogenesis, much attention was paid to clarify/identify the possible neural routes of entry and ways of dissemination of

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**Fig. 1.** A. Scuticociliates (arrows) inside the nerve bundles (N. B.) in the muscle tissue (M). B. Scuticociliates (arrows) in the nerve endings (N. End.) in the muscle tissue (M). Muscle tissue from infected fish taken on day 2 post-infection. Bar=100 μm.

**Fig. 2.** A. Eye (E) showing severe infection of the scuticociliates (arrows) in the periorbital cavity (Per. C.). Bar=50 μm. B. Optic nerve (Opt. N.) showing scuticociliates (arrows) with inflammatory changes. Optic nerve from infected fish taken on day 3 post-infection. Bar=50 μm.
the scuticociliate in the host tissue. The same experimental design was used by Miwa and Nakayasu [14].

Few mortality cases were observed in the control group till day 10 post-infection. This might be due to some stress factors, lack of nutrients, and/or some infectious agents either bacteria or parasite. Some fish were found to be infected individually with ciliates which were PCR negative for Miamiensis avidus. However, the scuticociliate infected groups showed higher mortalities than the control group.

In the present study, the scuticociliates were firstly observed in the skin and/or gills from day 0 post-infection and then disseminate to different body tissues, suggesting that the first possible invasion route may be the dermal and/or branchial epithelium (skin and/or gill) [4, 9, 11, 16].

Whereas, the scuticociliates were frequently observed along the optic and/or olfactory nerve in the fish which were accompanied by severe brain lesions but by minimum lesions in the gills and skin. The findings might suggest that in addition to skin and/or gills, neural routes including the periorbital and nasal routes may play a role in the current scuticociliate invasion to the brain [15, 16].

Extensive epidermal damage, dermal degeneration, hyperplasia of branchial epithelium, necrotic myositis, encephalitis and myelitis were observed in the present study; these changes are in agreement with previous studies on the pathology of the outbreaks and experimental cases of scuticociliatosis in fish [1, 7, 9–11]. Brain infection was observed in most of the fish examined, suggesting that the brain is one of the most susceptible organs. Pathological examination of the dead fish taken from day 6 post-infection onwards, when a sudden leap in mortality detected, showed extensive damage in the brain without severe lesions in the other tissues and organs. These findings suggest the significance of the brain lesions as the cause of death of the infected fish.

Blood stream has been regarded as a major way of dissemination into the other tissues [9, 11, 16, 18, 22]. In addition, the connective tissue was previously suggested as a possible quick way of dissemination of the scuticociliate to the body [17]. In the present study, the scuticociliates were observed in and along the peripheral, spinal and cranial nerve fibers, cranial cavity and in the vertebral canal, suggesting that the ciliates could spread from the entry sites not only through blood stream and connective tissue but also through nerve fibers and/or cerebrospinal fluid circulation.

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