Histopathology of *Aphanomyces* Infection in Dwarf Gourami (*Colisa lalia*)

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Histopathological study was made on an epizootic of systemic mycosis due to *Aphanomyces* sp. in an ornamental fish species, dwarf gourami (*Colisa lalia*), imported from Singapore in 1991 and 1992. In almost all cases, the external clinical signs in infected fish included erosions and/or ulcers on the body surface, and swelling of the abdomen. Histopathological examination of the fish revealed that the disease was characterized by systemic granulomatous lesions associated with *Aphanomyces* sp.

In this paper, we describe detailed histopathological features of the mycotic lesions and compared the features with those of other mycoses, such as mycotic granulomatosis, ulcerative mycosis, red spot disease and epizootic ulcerative syndrome. The present case shared similar histopathological features including mycotic granulomatous lesions, with those of earlier reported mycoses.

Recently, an outbreak of *Aphanomyces* infection was found in ornamental fish, dwarf gourami (*Colisa lalia*), imported from Singapore (Hatai *et al.*, 1994). Histopathologically, the disease was characterized by systemic granulomatous lesions associated with *Aphanomyces* sp. In Japan, intense granulomatous responses to invading fungi, morphologically consistent with oomycetes, have also been described in mycotic granulomatosis of ayu, *Plecoglossus altivelis* (Egusa and Masuda, 1971; Miyazaki and Egusa, 1973a), goldfish, *Carassius auratus* (Miyazaki and Egusa, 1972), bluegill, *Lepomis macrochirus* (Miyazaki and Egusa, 1973b), and some wild fish (Kumamaru, 1973; Miyazaki and Egusa, 1973c). Noga *et al.* (1988) described similar inflammatory responses in ulcerative mycosis of Atlantic menhaden, *Brevoortia tyrannus*. Such inflammatory responses were also reported in red spot disease of sea mullet, *Mugil cephalus* (Callinan *et al.*, 1989), and in epizootic ulcerative syndrome (EUS) of many kinds of freshwater and estuarine fish (Lilley *et al.*, 1992; Roberts *et al.*, 1993). In this paper, the authors report detailed histopathological features of the systemic aphanomycosis in dwarf gourami, and compare the characteristics with those of mycotic granulomatosis, ulcerative mycosis, red spot disease and EUS cases.

Materials and Methods

A total of 36 fish ranging from 1.7 to 3.2 g in body weight and 3.3 to 4.2 cm in body length were collected from pet shops in Tokyo from September 1991 to February 1992. After observation of the gross findings, a necropsy was routinely performed. A small piece of trunk muscle and/or gonad was cut from the fish for microscopical examination. The fish were fixed in 10% phosphate-buffered formalin solution, embedded in paraffin, and sectioned at 4 to 5 μ m. For each fish examined, we prepared five to six paraffin sections as follows; horizontal sections of the second gill arches of both sides, longitudinal section of the head which was cut off just behind the opercula, longitudinal section of the visceral organs, transverse section of the kidney which was cut off at the middle of the trunk, and the skin and trunk muscles of which contain erosious and/or ulcerative lesions at the body surface. Sections were stained with methenamine silver-nitrate-Grocott's variation with haematoxylin and eosin (Grocott-H & E). Some sections were also stained with the Ziehl-Neelsen (ZN) method for acid-fast bacteria, Giemsa, and periodic acid Schiff (PAS) reaction.

In order to evaluate the histological findings, numbers of hyphae found in the trunk muscles, abdominal adipose tissue, pancreas, kidney, liver, gonad,

head kidney, spleen, central nervous system, alimentary tract, and heart were counted under light microscopy. The longitudinal sections of the visceral organs were used to examine the liver, spleen, alimentary tract, gonad, abdominal adipose tissue and pancreas. The heart, head kidney, and central nervous system were examined by using of the longitudinal sections of the head. For the counting of hyphae, 4 to 10 fields were examined under 200 magnifications depending on the individual organ size. From these data, the average number of hyphae in one microscopical field of each organ was calculated. Then, each organ of individual fish was classified into four groups according to the average number of hyphae as follows; 1^+ with one to three hyphae, 2^+ with four to six hyphae, 3^+ with seven to nine hyphae, and 4^+ with over 10 hyphae in one field. With these scores of the degree, average score of fungal invasion (Ave = a total score of the degrees in each organ/number of examined specimens) and incidence of mycotic lesion ($Inc\%$ = number of specimens with mycotic lesions/number of examined specimens $\times 100$) of each organ were calculated. Finally, the severity of mycotic lesion (SML) value of each organ was calculated with Ave and $Inc\%$ ($SML = Ave \times Inc\%$) (Table 1).

Results

Gross findings

The cutaneous lesions found in the examined fish were classified into two types based on the gross findings. Type 1 lesions were found in almost all fish examined; these were small discolored pale areas, 3 to 10 mm in diameter, with irregular reddening. Scales were frequently absent from the lesion centers and the peripheral portions of the lesions were erosive (Fig. 1). In 30 out of the 36 fish, Type 2 lesions were observed. The lesions consisted of open ulcers up to 10 mm in diameter. The central necrotic core of tissue sloughed off, leaving a crater-shaped cavity that at times was surrounded by an erosive periphery. These necrotic craters usually exposed the trunk muscles (Fig. 1).

In almost all cases, asymmetrical swelling of the abdomen without accumulation of ascitic fluid was observed (Fig. 2). In such fish, the abdominal wall was slightly adhered to the internal organs. These fish also showed discoloration of the liver, swelling of the liver and kidney, and irregular reddening of the

Fig. 1. Lateral view of an affected fish. Note erosive Type 1 lesion (arrow head) and ulcerative Type 2 lesion (arrow). Bar = 1.0 cm.

Fig. 2. Dorsal view of an affected fish. Note asymmetrical swelling of the abdomen (arrow). Bar = 1.0 cm.

Fig. 3. Numerous fungal hyphae found in the trunk muscle. The hyphae were partially surrounded by dark brown sheaths (arrows). Unfixed and unstained, bar = 100 μ m.

gonad. Furthermore, some of these fish had small white spots in the liver, kidney and gonad.

Microscopical examination of the unfixed trunk muscles and gonad revealed numerous fungal hyphae, which were irregularly branching, aseptate and approximately 13 μ m in diameter, intertwined with necrotic tissue (Fig. 3). These hyphae were partially surrounded by dark brown sheaths, morphologically consistent with granulomatous tissue (Fig. 3).

Histopathological findings

1) Lesions in cutaneous, muscular and bone tissues

Histopathologically, tissue reactions consisted of intense chronic inflammation associated with the formation of granulomas without any ectoparasites. In Type 1 lesions, which had discoloration, scale loss and erosion in the skin, the epidermis and stratum spongiosum sloughed off, leaving the degenerated stratum compactum (Fig. 4). Granulomas were found from the stratum compactum to the muscle layer. These granulomas consisted of epithelioid cells, and were occasionally surrounded by a thin rim of fibroblasts (Fig. 5a). Multinuclear giant cells were not observed in the granulomatous lesions. Large numbers of sparsely branching, aseptate fungal hyphae of 8 to 20 μm in diameter, morphologically consistent with an oomycete fungus, were usually present within the granulomas (Fig. 5a). The hyphae were most easily recognized in Grocott-H&E preparations, although they were also stained positively with PAS reaction. Large granulomas had necrotic, eosinophilic debris surrounding the hyphae in the center of the granulomas. These debris showed similar dye-affinity to the skeletal muscles by Grocott-H&E and PAS reaction. In some of the

granulomas, the epithelioid cells showed degeneration and necrosis containing large vacuoles in the cytoplasm (Fig. 5b). Discrete granulomas were surrounded by a loose fibrous matrix containing fibroblasts, macrophages and a few eosinophilic granulocytes (Fig. 5c). The matrix appeared to follow myomeres and to encapsulate the necrotized muscle fibers (Fig. 5c). In fish with slight fungal infection, the fungal hyphae were merely encapsulated by such a matrix, and the matrix of severely infected fish also had a few fungal hyphae. From these findings, it seemed likely that the granulomas with hyphae were generated from cellular contents in such a matrix. There was severe floccular degeneration and necrosis of skeletal muscle fibers (Fig. 5d). The ZN method for acid-fast organisms and Giemsa stain did not reveal any other microorganisms in Type 1 lesions.

In ulcerative lesions which were classified into Type 2, large amounts of tissue from the epidermis to the stratum compactum sloughed off, leaving necrotic skeletal muscle layer with numerous mycotic granulomas (Fig. 6). The granulomas were found in the deeper layer of skeletal muscles, and fungal hyphae, morphologically similar to that found in Type 1 lesions, often penetrated into vertebrae and costae

Fig. 4. Low magnification of Type 1 lesion. The epidermis and stratum spongiosum sloughed off, leaving the degenerated stratum compactum (arrow). The muscle layer was replaced by many mycotic granulomas (arrow heads) and inflammatory cells. Grocott-H&E, bar = 200 μm .

Fig. 5. Detailed histopathological features of mycotic granulomatous lesions.

a; Each granuloma consisted of epithelioid cells with a thin rim of fibroblasts (arrow). Numerous fungal hyphae were found within the granulomas (arrow heads). Grocott-H&E, bar=50 μ m. b; Note degenerative epithelioid cells containing large vacuoles in the cytoplasm (arrow). Grocott-H&E, bar=50 μ m. c; Discrete granulomas were surrounded by a loose fibrous matrix associated with myomeres (arrows). The matrix appeared to encapsulate the necrotized muscle fibers (arrow heads). Grocott-H&E, bar=100 μ m. d; Note severe floccular degeneration and necrosis of skeletal muscle fiber (arrows). Grocott-H&E, bar=200 μ m.

(Figs. 7a, b) in 20 of the fish examined. Multinuclear giant cells resembling osteoclasts were not observed in such lesions. Loose edematous fibrous tissue with a few macrophages was found between the degenerated and necrotic skeletal muscle fibers. Bacteria were not found in the deeper layer of the skeletal muscles, although Giemsa stain revealed moderate bacterial invasion in the surface layer of the exposed skeletal muscles. Some acid-fast microorganisms could be detected in Type 2 lesions by the ZN method. However, these ulcerative lesions were not found in fish with slight fungal invasion.

2) Lesions in internal organs

Advanced lesions extended deep into the body and thus involved adjacent internal organs. Lesions overlying the peritoneal cavity almost invariably affected the viscera by extension. The abdominal

adipose tissue (Fig. 8), pancreas, kidney, liver, gonad, head kidney, spleen, alimentary tract and heart could all be affected with mycotic granulomas. The granulomas in such organs showed similar findings to those in the cutaneous and muscular lesions. Small white spots found in the liver, kidney, and gonad were histologically recognized as granulomatous lesions surrounding hyphae.

In 10 of the fish examined, their central nervous systems were also affected by hyphae. Although almost all granulomatous lesions in the spinal cords and brains were similar to those in other organs (Figs. 9a, b), a few hyphae in such organs merely caused feeble inflammatory responses consisting of a few small rounded cells resembling neuroglia and fibroblasts (Fig. 9c). Further, hyphae without any cellular responses were also found in some brains

Fig. 6. Low magnification of Type 2 lesion. Remnants of the stratum compactum (arrows) overlie the lesion. Note the necrotic skeletal muscle layer with numerous mycotic granulomas (arrow heads). Grocott-H & E, bar = 100 μ m.

Fig. 7. Hyphae invaded into the bony tissues (arrows heads).
a; vertebra. Grocott-H & E, bar = 100 μ m. b; costa. Grocott-H & E, bar = 100 μ m.

Fig. 8. Mycotic lesions in the abdominal adipose tissue. Numerous fungal hyphae with granulomatous sheaths (arrows) scattered in the tissue. Grocott-H & E, bar = 200 μ m.

Fig. 9

Table 1. Summary of the histopathological findings in this study

No.	TM.	AAT.	Panc.	Kid.	Liv.	Gon.	HK.	Spl.	CNS.	AT.	Heart	Total
1	1 ⁺	—* ¹	—	—	—	—	—	—	—	—	—	1
2	4 ⁺	—	—	—	—	—	—	—	—	—	—	4
3	4 ⁺	2 ⁺	—	1 ⁺	—	—	—	—	—	—	—	7
4	4 ⁺	1 ⁺	1 ⁺	1 ⁺	—	—	—	—	—	—	ND* ²	7
5	4 ⁺	—	—	3 ⁺	—	—	2 ⁺	—	1 ⁺	—	—	10
6	4 ⁺	2 ⁺	2 ⁺	1 ⁺	1 ⁺	—	ND	—	—	—	—	10
7	4 ⁺	2 ⁺	1 ⁺	2 ⁺	1 ⁺	1 ⁺	—	—	—	—	—	11
8	4 ⁺	3 ⁺	2 ⁺	—	1 ⁺	—	—	1 ⁺	—	—	—	11
9	4 ⁺	2 ⁺	1 ⁺	1 ⁺	—	1 ⁺	1 ⁺	1 ⁺	—	—	—	11
10	4 ⁺	4 ⁺	2 ⁺	—	—	2 ⁺	—	—	—	1 ⁺	—	13
11	4 ⁺	3 ⁺	3 ⁺	ND	—	—	—	3 ⁺	ND	—	ND	13
12	4 ⁺	2 ⁺	2 ⁺	4 ⁺	—	—	—	—	2 ⁺	—	—	14
13	4 ⁺	3 ⁺	3 ⁺	ND	2 ⁺	—	1 ⁺	—	—	—	1 ⁺	14
14	4 ⁺	2 ⁺	1 ⁺	2 ⁺	—	1 ⁺	2 ⁺	3 ⁺	—	—	ND	15
15	4 ⁺	3 ⁺	2 ⁺	1 ⁺	1 ⁺	2 ⁺	2 ⁺	1 ⁺	—	—	—	16
16	4 ⁺	2 ⁺	2 ⁺	3 ⁺	2 ⁺	—	1 ⁺	1 ⁺	2 ⁺	—	ND	17
17	4 ⁺	3 ⁺	2 ⁺	4 ⁺	2 ⁺	1 ⁺	2 ⁺	—	—	—	—	18
18	4 ⁺	3 ⁺	—	4 ⁺	2 ⁺	ND	4 ⁺	—	2 ⁺	—	—	19
19	4 ⁺	3 ⁺	3 ⁺	3 ⁺	2 ⁺	4 ⁺	—	1 ⁺	—	—	—	20
20	4 ⁺	3 ⁺	3 ⁺	4 ⁺	1 ⁺	4 ⁺	1 ⁺	—	—	—	ND	20
21	4 ⁺	3 ⁺	2 ⁺	2 ⁺	3 ⁺	4 ⁺	1 ⁺	1 ⁺	—	—	—	20
22	4 ⁺	4 ⁺	3 ⁺	3 ⁺	1 ⁺	4 ⁺	1 ⁺	1 ⁺	—	—	—	21
23	4 ⁺	4 ⁺	4 ⁺	1 ⁺	4 ⁺	ND	1 ⁺	1 ⁺	2 ⁺	—	—	21
24	4 ⁺	4 ⁺	4 ⁺	2 ⁺	3 ⁺	4 ⁺	ND	ND	—	—	—	21
25	4 ⁺	4 ⁺	3 ⁺	2 ⁺	2 ⁺	4 ⁺	—	4 ⁺	—	—	ND	23
26	4 ⁺	4 ⁺	4 ⁺	4 ⁺	1 ⁺	4 ⁺	—	1 ⁺	1 ⁺	—	—	23
27	4 ⁺	4 ⁺	3 ⁺	2 ⁺	2 ⁺	2 ⁺	2 ⁺	3 ⁺	1 ⁺	—	—	23
28	4 ⁺	4 ⁺	4 ⁺	1 ⁺	3 ⁺	4 ⁺	—	4 ⁺	—	—	—	24
29	4 ⁺	4 ⁺	4 ⁺	2 ⁺	4 ⁺	2 ⁺	ND	2 ⁺	—	3 ⁺	ND	25
30	4 ⁺	4 ⁺	4 ⁺	4 ⁺	2 ⁺	4 ⁺	ND	4 ⁺	—	—	—	26
31	4 ⁺	4 ⁺	4 ⁺	2 ⁺	2 ⁺	4 ⁺	3 ⁺	4 ⁺	—	—	—	27
32	4 ⁺	4 ⁺	4 ⁺	3 ⁺	2 ⁺	4 ⁺	4 ⁺	2 ⁺	—	—	ND	27
33	4 ⁺	4 ⁺	4 ⁺	4 ⁺	1 ⁺	1 ⁺	4 ⁺	4 ⁺	—	2 ⁺	—	28
34	4 ⁺	4 ⁺	4 ⁺	3 ⁺	2 ⁺	4 ⁺	2 ⁺	4 ⁺	1 ⁺	—	—	28
35	4 ⁺	4 ⁺	4 ⁺	3 ⁺	4 ⁺	4 ⁺	4 ⁺	2 ⁺	2 ⁺	—	ND	31
36	4 ⁺	4 ⁺	4 ⁺	4 ⁺	3 ⁺	4 ⁺	4 ⁺	4 ⁺	1 ⁺	2 ⁺	ND	34
Total	141	106	89	76	54	69	42	52	15	8	1	
n=	36	36	36	34	36	34	32	35	35	36	26	
Ave* ³	3.9	2.9	2.4	2.2	1.5	2.0	1.3	1.5	0.4	0.2	0.04	
Inc %* ⁴	100.0	91.7	86.1	94.1	72.2	67.6	59.4	62.9	28.6	11.1	3.8	
SML* ⁵	390.0	265.9	206.6	207.0	108.3	135.2	77.2	94.4	11.4	2.2	0.2	

TM.; trunk muscle, AAT.; abdominal adipose tissue, Panc.; pancreas, Kid.; kidney, Liv.; liver, Gon.; gonad, HK.; head kidney, Spl.; spleen, CNS.; central nervous system, AT.; alimentary tract.

*¹ Hyphae were not observed in the histological section; *² The organ was not found in the histological sections;

*³ Average score of the degree of fungal growth (total/n); *⁴ Incidence of mycotic lesions (numbers of specimens with mycotic lesions/numbers of specimens examined × 100); *⁵ Severity of mycotic lesions (Ave. × Inc %).

Fig. 9. Mycotic granulomatous lesions in the central nervous system.

a; Matured mycotic granuloma consisted of epithelioid cells in the spinal cord. Grocott-H&E, bar = 50 μ m. b; Early stage of mycotic granuloma in the brain with a few epithelioid cell layers and infiltration of small rounded cells. Grocott-H&E, bar = 30 μ m. c; Feeble inflammatory responses consisting of a few small rounded cells (arrow) around the hypha penetrated into the spinal cord. Grocott-H&E, bar = 50 μ m. d; Penetrating hyphae (arrows) in the brain without any cellular response. Grocott-H&E, bar = 100 μ m.

examined (Fig. 9d). A small number of granulomas with acid-fast microorganisms were observed in the kidney, head kidney and spleen of some fish with slight fungal invasion.

SML values could be arranged in decreasing order as follows; the trunk muscle, abdominal adipose tissue, kidney, pancreas, gonad, liver, spleen, head kidney, central nervous system, alimentary tract and heart (Table 1).

Discussion

Histopathologically, the present case was characterized by intense granulomatous lesions associated with a oomycete fungus, and the causative fungus was morphologically consistent with *Aphanomyces* sp. isolated from the same specimens used in this paper (Hatai *et al.*, 1994). Intense granulomatous lesions associated with oomycete fungi were previously reported in mycotic granulomatosis (MG) in many kinds of fish (Egusa and Masuda, 1971; Miyazaki and Egusa, 1972; Kumamaru, 1973; Miyazaki and Egusa, 1973a, b, c), ulcerative mycosis (UM) (Noga *et al.*, 1988), red spot disease (RSD) (Callinan *et al.*, 1989), and in epizootic ulcerative syndrome (EUS) (Lilley *et al.*, 1992; Roberts *et al.*, 1993). The granulomatous responses found in the present case were similar to those of MG in goldfish (Miyazaki and Egusa, 1972), UM in Atlantic menhaden (Noga *et al.*, 1988), RSD in sea mullet (Callinan *et al.*, 1989) and EUS in many kinds of fresh water and estuarine fish (Lilley *et al.*, 1992; Roberts *et al.*, 1993). The absence of multinuclear giant cells in the present lesions was similar to MG in snakehead, *Channa argus*, and sea mullet (Miyazaki and Egusa, 1973c) and RSD in sea mullet (Callinan *et al.*, 1989). In addition, fungi belonging to the genus *Aphanomyces* were isolated from almost all previous cases (Hatai *et al.*, 1977; Dykstra *et al.*, 1986; Fraser *et al.*, 1992; Roberts *et al.*, 1993) and all of the authors concluded that isolated fungi might act as important etiological agents in the pathogenesis of the cases. Therefore, the present case should be classified into a category of mycotic granulomatous diseases resembling MG, UM, RSD and EUS.

The histopathological findings and the decreasing order of SML values mentioned above suggested that the fungus invaded from the body surface, established the primary lesion at the trunk muscle and then hyphae penetrated into the peritoneal cavity

inducing granulomatous inflammation in the adjacent organs. Most of previous reports also described that hyphae would invade from the body surfaces and penetrate into the peritoneal cavities (Miyazaki and Egusa, 1972; Miyazaki and Egusa, 1973a, b, c; Noga *et al.*, 1988; Callinan *et al.*, 1989; Roberts *et al.*, 1993). The Type 1 and Type 2 lesions described in this article are considered to be consistent with "moderate lesion and severe lesion" in MG of goldfish (Miyazaki and Egusa, 1972), "Early Type II lesion and Advanced lesion" in UM of Atlantic menhaden (Noga *et al.*, 1988), and "Necrotizing dermatitis and Dermal ulcer" in RSD of sea mullet (Callinan *et al.*, 1989), respectively. All of these previous reports suggested that the formers were transitional forms, consistent with evolution of these lesions to the later. In the light of these suggestions, the Type 1 lesions were considered to precede the Type 2 lesions. We did not observe any other microorganisms under the light microscope in the Type 1 lesions and hence these microorganisms were not considered the primary agent inducing fungal infection in the present case.

Miyazaki and Egusa (1972) reported MG in goldfish with lesions in the brain and spinal cord. Most lesions in the central nervous systems found in the present case were similar to those in the goldfish case, as were granulomatous responses in spinal cords and feeble inflammatory responses in brains against penetrating hyphae, although the present case had some mycotic lesions without any cellular responses in the brains. In the present case, the epithelioid cells surrounding hyphae sometimes showed degeneration and necrosis, and destruction of bony tissues by penetrated hyphae were also observed. These findings were considered to suggest intense invasiveness of the hyphae beyond self-defense mechanisms of the host.

EUS occurring in south-east Asia, including Singapore where the examined fish in the present study were reproduced, has not been determined as its primary etiological agent. However, Roberts *et al.* (1993) reported the specific slow-growing, thermolabile *Aphanomyces* isolated from the fish affected with EUS. They described that the isolate was the pathogenic fungus which caused so much tissue damage in EUS. The histopathological findings of the present case were similar to those of EUS (Lilley *et al.*, 1992; Roberts *et al.*, 1993). It is noteworthy if the present case is regarded as EUS, though it needs

further studies regarding the pathogenicity and the detailed biological characteristics of the fungi isolated from the present case and EUS.

No preventive systems have been devised against spreading new pathogens introduced with imported tropical ornamental fish as described in the present study. In Japan, when MG in ayu prevailed, the outbreak of an epizootic with similar symptoms to MG occurred in 20 kinds of feral fish species at Lake Kasumigaura and Lake Kitaura, Ibaraki Prefecture in 1971 and 1972 (Kumamaru, 1973). From these findings, it is suggested that once foreign pathogenic *Aphanomyces* strains are introduced in domestic freshwater areas, it might affect cultured and feral fish in Japan. Studies are presently underway to determine if experimental infection with the fungus isolated from the present case can produce the disease in some cultured fish in Japan.

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