Fluorochrome Uvitex 2B Stain for Detection of the Microsporidian Causing Boko Disease of Yellowtail and Goldstriped Amberjack Juveniles

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The fluorochrome Uvitex 2B [4,4-BIS(2-di(2-hydroxyethyl)-amino-4-(3-sulfophenylamino)-1,3,5-triazine-6-ylamino)-stilbene-2,2-disulfonic acid, sodium salt] stain was used for detection of *Microsporidium seriolae* (Protozoa: Microspora), the causative agent of boko disease of yellowtail *Seriola quinqueradiata* and goldstriped amberjack *S. lalandi* juveniles. For detection of the parasite, microscopical fluorescence examination for spores in the Uvitex 2B-stained smears of trunk muscle homogenates was much more sensitive than the conventional visual inspection for "cysts" in the trunk muscle. The Uvitex 2B-H & E stain of deparaffinized sections was applicable to the examination for the sporulation sequence in "cysts" and spore dispersal into adjacent tissues or other organs. Thus, Uvitex 2B stain was found to be useful not only for a rapid and sensitive diagnosis of boko disease but also for histopathological studies of microsporidian infections.

Key words: Uvitex 2B, *Microsporidium seriolae*, diagnosis, microsporidian, yellowtail, goldstriped amberjack, fluorochrome stain

"Boko" disease in yellowtail *Seriola quinqueradiata* and goldstriped amberjack *Seriola lalandi*, characterized by concave depressions of the body surface of infected fish, often leads the fish to death and produces serious damages in seed productions of them. The causative pathogen, *Microsporidium seriolae* (Protozoa: Microspora), forms macroscopic cyst-like bodies containing various parasite stages in the trunk muscle of the host fish (Egusa, 1982).

Microsporidians are intracellular protozoan parasites that have been documented to infect most animal groups of both invertebrates and vertebrates, including fish, insects, and mammals (Canning and Lom, 1986). Recently, human microsporidiosis in patients with HIV have been recognized (Desportes *et al.*, 1985; Cali *et al.*, 1993) and some new diagnostic techniques have been developed. The staining with the fluorochrome Uvitex 2B [4,4-BIS(2-di(2-

hydroxyethyl)-amino-4-(3-sulfophenylamino)-1,3,5-triazine-6-ylamino)-stilbene-2,2-disulfonic acid, sodium salt], which binds to chitin, a component of the microsporidian spore wall, was successfully applied to detection of microsporidian *Enterocytozoon bienersi* and *Septata intestinalis* in smears of stool and duodenal aspirates from AIDS patients (Van Gool *et al.*, 1993; DeGirolami *et al.*, 1995).

Our purpose of the present study is to develop a new detection method for the microsporidian *M. seriolae* from fish tissues using the Uvitex 2B stain.

Materials and Methods

Hatchery-reared yellowtail and goldstriped amberjack juveniles were transported to netpens at Goto Station of Japan Sea-Farming Association (20 June 1995) and Nagasaki Prefectural Fisheries Experi-

mental Station (30 June 1995), respectively. Occurrences of *Microsporidium seriolae* infection were subsequently monitored. Randomly selected fish were filleted and visually inspected for the presence of microsporidian "cysts" in the trunk muscle. The

samples were stored at -20°C and later, examined microscopically as follows. After the samples were defrozen, pieces of the trunk muscle (about 1 g) were homogenized with physiological saline, and passed through a steel mesh sieve (Sigma, No. 40), then

Figs. 1-3. Smear of trunk muscle homogenate from goldstriped amberjack infected with *Microsporidium seriolae*. Uvitex 2B stain. **1;** tissue debris observed with normal light. **2;** spores visualized with UV light in the same field as Fig. 1. **3;** typical ovoidal spores recognized at a higher magnification. Scale bars are $20\mu\text{m}$ in **1** and **2**, $10\mu\text{m}$ in **3**.

centrifuged at $500 \times g$ for 10 min. After the supernatant was discarded, the pellets were collected by a swab and smeared onto a circular area (about 15 mm in diameter) on a glass slide. After fixation with methanol, air-dried slides were stained with Uvitex 2B according to DeGirolami *et al.* (1995); briefly, the preparates were covered with 1% Uvitex 2B (Ciba Geigy, Switzerland) solution dissolved in phosphate buffered saline (PBS) for 10 min, then counterstained with 0.5% Evans Blue solution in PBS for 30 sec. After rinsed with distilled water, air-dried smears were examined for the presence of spores with a fluorescence microscope equipped with a 100 W mercury bulb (ultra violet excitation; 330–380 nm). The kidney and heart were also removed from each fish, bisected and impressed onto a glass slide. The impression smears of the kidney and heart were examined for the spores using the Uvitex 2B stain as described above.

For histological observations, pieces of the muscle, kidney, heart, gill, stomach and intestine of infected and control fish were fixed in 10% formalin or Bouin's solution, embedded in paraffin, then sectioned at $5 \mu\text{m}$. Deparaffinized sections were stained with Uvitex 2B, subsequently with H&E, then observed with a fluorescence microscope (UV and/or normal light).

Results

In the Uvitex 2B-stained smears, each *Microsporidium seriolae* spore was clearly recognized as an ovoid body with bright white fluorescence at a magnification of $\times 200$ and easily distinguishable from the other fluorescein contaminants on the basis of the morphological characteristics ($2\text{--}4 \mu\text{m}$ in size and ovoid in shape) at a magnification of $\times 400$ (Figs. 1–3). The Uvitex 2B staining method with smears of trunk muscle homogenates made it possible to detect even a few spores in light infections which could not be detected by visual inspection for “cysts”.

The detection rates by Uvitex 2B staining of trunk muscle homogenates were much higher than those by visual inspection in all cases tested in this study; on July 20, 7 out of 50 amberjack samples were positive for “cysts” in the trunk muscle, while 13 out of 50 were positive for Uvitex 2B-stained spores (Table 1). In the case of yellowtail, “cysts” in the trunk muscle were first found on July 20, while Uvitex 2B-stained spores were detectable in earlier periods (Table 2).

Table 1. Comparison of detection for *Microsporidium seriolae* in goldstriped amberjack juveniles between visual inspections and microscopical examinations by Uvitex 2B staining

Date	Detection rate*			
	Visual “cysts” in trunk muscle	Spores stained with Uvitex 2B in trunk muscle	in kidney	in heart
1995				
Jul. 4	0/50	6/50	—**	—
Jul. 20	7/50	13/50	2/50	0/50

*: Number of fish in which “cysts” or spores was detected/number of fish examined.

** : Not done.

Table 2. Comparison of detection for *Microsporidium seriolae* in yellowtail juveniles between visual inspections and microscopical examinations by Uvitex 2B staining

Date	Detection rate*			
	Visual “cysts” in trunk muscle	Spores stained with Uvitex 2B in trunk muscle	in kidney	in heart
1995				
Jun. 30	0/60	1/20	—**	—
Jul. 10	0/60	2/20	0/20	0/20
Jul. 20	15/60	10/20	3/20	2/20
Jul. 30	50/60	19/20	7/20	3/20
Aug. 10	57/60	20/20	14/20	10/20
Aug. 21	52/60	19/20	11/20	9/20
Aug. 31	48/60	17/20	8/20	8/20
Sep. 11	47/60	20/20	11/20	12/20

*: Number of fish in which “cysts” or spores was detected/number of fish examined.

** : Not done.

The detection rate for spores in the trunk muscle was much higher than those in the kidney and the heart, in which smaller numbers of spores were detected. In the periodical sampling of yellowtail, a time lag was noted in the time of increment of detection rate between the trunk muscle and other organs (kidney and heart); the detection rate in the trunk muscle rose between July 10 and July 30, while those in the kidney and the heart increased between July 30 and August 10 (Table 2).

In the histological sections, a sporulation sequence was distinctly recognized in *Microsporidium* “cysts”, which were composed of Uvitex 2B-negative multinucleate plasmodia in the periphery and positive

spores in the center (Fig. 4). After disintegration of "cysts", spores were dispersed from the ruptured "cysts" to the surrounding area. Most of liberated spores were ingested by phagocytes, and these phago-

cytes formed melanomacrophage centers (MMCs) in the adjacent muscle tissues (Fig. 5). Some intact or phagocytosed spores were also observed in the subcutaneous, dermal and epidermal tissues near the

Figs. 4-7. Histological sections of yellowtail infected with *Microsporidium seriolae*. Uvitex 2B-H&E stain. Double exposure with UV and normal light. **4;** trunk muscle "cysts" composed of Uvitex 2B-positive spores and negative immature stages. **5;** spores liberated into surrounding tissues. Some spores are engulfed by phagocytes. Melanin deposition is observed. **6;** spores in the kidney MMC. **7;** spores in the lumen of ventricle. Scale bars are 100 μm in **4**, 50 μm in **5**, 10 μm in **6** and **7**.

disintegrated "cysts" in the muscle. Spores were also detected in other organs such as the heart, intestine, kidney and gill lamellae. Spores observed in the kidney were mostly located in the MMCs (Fig. 6). A few spores, which were phagocytosed or intact, were found in the intestinal and ventricle lumina (Fig. 7). Immature developmental stages were not observed in organs other than the trunk muscle.

Discussion

Detection of microsporidian with conventional microscopic method using H & E and/or Giemsa has been sometimes difficult, particularly in light infections, because of a poor staining selectivity for spores. Definitive diagnosis has often to depend on time-consuming and labor-intensive methods such as TEM. On the other hand, the Uvitex 2B-staining method was found to have a high staining selectivity for microsporidian spores and have some advantages and limitations as follows.

Advantages: 1) Detection of microsporidian spores is rapid (about 1 h for the whole procedure in case of smears) and easy even at a low magnification ($\times 200$) of a fluorescein microscope.

2) Double staining with H & E in deparaffinized sections is possible.

Limitations: 1) Detection of immature stages which have not yet produced a chitinous wall, is impossible.

2) Different species of microsporidian can not be differentiated.

Thus, Uvitex 2B stain is useful not only for a rapid and sensitive diagnosis of microsporidiosis but also for histopathological studies of microsporidian infection. For the purpose of early diagnosis of specific microsporidian, the other method such as immunological method using specific antibodies and PCR method might be established.

Awakura (1974) reported that *Microsporidium takedai* formed "cysts" in the heart muscle more frequently than in the trunk muscle, particularly in chronic cases in salmonid fish. However, in the present study, *Microsporidium seriolae* developed sporogonial forms in the trunk muscle of yellowtail and goldstriped amberjack, and "cyst" formation in the other organ was not observed.

In case of *Myxobolus artus* (Myxozoa: Myxosporidia) developing in "cysts" in the trunk muscle of common carp *Cyprinus carpio*, the spore recovery

rate from the kidney was markedly higher than the detection rate for "cysts" by visual inspections of the trunk muscle (Ogawa *et al.*, 1992; Yokoyama *et al.*, 1996). After disintegration of "cysts" in the trunk muscle, quite a few *M. artus* spores were transported to other organs by host's macrophages, possibly through the vascular system, and massive accumulation of spores in the kidney MMCs made the detection of parasites so sensitive. *Microsporidium seriolae* spores were also spread to other organs, probably due to process of host response similar to *Myxobolus artus* infection in carp. The presence of phagocytosed spores in the ventricle lumina and a later increase of the detection rate of spores in the heart and the kidney supports this idea. However, the spore transportation appears to occur in substantially smaller degree than that in *Myxobolus artus* infection in carp.

Presence of *Microsporidium seriolae* spores in the dermal and epidermal tissues suggests that the spores might be released outside of the body via skin. Delgahapitiya (1994) investigated the development of *Heterosporis anguillarum*, the causative microsporidian of beko disease of Japanese eel *Anguilla japonica* with a special emphasis on the host responses, and described a phenomena of spore movement to the skin and to the other organs of eel similar to that in the present study. On the other hand, *H. anguillarum* did not form distinct "cysts", although sporogonial forms were recognized as milky-white patches in the musculature.

Although these protozoan parasites resemble each other in the mode of development, especially in the appearance of sporogonial forms in the trunk muscle of host fish, the differences mentioned above suggest that the host-parasite relationship are different between them. It is possible that the differences may be caused by different in host and/or parasite species. Further studies on host-parasite relationships in each infection are needed.

In the present study, we showed that the Uvitex 2B staining method provided a rapid and sensitive diagnosis for Beko disease in yellowtail and amberjack juveniles. As Uvitex 2B specifically bind to chitin, a common component of microsporidian spores, this method could be applicable to the diagnosis and studies of other microsporidians as well.

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