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Original Article

Diplomonad flagellates of some ornamental fish cultured in Thailand

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Abstract

The study on diplomonad flagellates infection in some ornamental fishes in the family cichlidae i.e., angelfish (*Pterophyllum scalare*), oscar (*Astronotus ocellatus*), blue mbuna (*Labeotropheus fuelleborni*) and the family osphronemidae i.e., Siamese fighting fish (*Betta splendens*) revealed that this parasite infected three out of four ornamental fish species, angelfish, oscar and blue mbuna. The highest infection was recorded in angelfish (90%) followed by oscar (75.4%) and blue mbuna (61%), respectively. Identification of diplomonad flagellates from angelfish by means of morphological studies under light and electron microscopes indicated that the parasite was *Spironucleus vortens*. The 14–days LD_{50} of *S. vortens* in angelfish was $2.99x10^3$ cells. Histopathological changes of infected angelfish revealed granulomatous liver, numerous numbers of melanomacrophage in the spleen and inflammation of the intestine. Susceptibility study of *S. vortens* to goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*) and platy (*Xiphophorus maculatus*) indicated that they were resistant to artificial infection. *In vitro* examination of the growth inhibition assay of *S. vortens* indicated that dimetridazole and metronidazole were effective in inhibiting parasite growth after 48 hrs exposure at concentrations of $\geq$4.0 µg/ml and $\geq$6.0 µg/ml, respectively. Magnesium sulfate at a concentration of $\geq$60 mg/ml inhibited the parasite growth after 72 hrs exposure. *In vivo* examination of the dimetridazole efficiency on *S. vortens* infection indicated that dimetridazole at 4.0 µg/ml provided the highest efficiency which could be used for treatment of spironucleosis in angelfish.

Keywords: Diplomonad flagellates, *Spironucleus vortens*, ornamental fish, treatment

1. Introduction

Infectious diseases pose a constant and serious threat to ornamental fish that are farmed intensively under conditions of high stocking density, poor water quality, and poor management. At present, various diseases have been reported in ornamental fish including bacterial diseases (Ferguson *et al.,* 1994; Pate *et al.,* 2005), viral diseases (Mellergaard and Bloch, 1988; Schuh and Shirley, 1990; Hedrick and McDowell, 1995), and parasitic diseases (Kim *et al.,* 2002a,b; Thilakaratne *et al.,* 2003; Leibowitz and Zilberg, 2009). Parasitic diseases of ornamental fish such as ichthyophthiriasis,

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trichodiniasis, and tetrahymeniasis are common (Kim *et al.*, 2002b; Tavares-Dias *et al.*, 2010). However, pathogenic intestinal protozoans including diplomonad flagellates are responsible for clinically important infections reported in many tropical fresh water fish species including anabantidae, belontidae, and cichlidae, and marine species, such as acanthuridae and pomacentridae (Becker, 1977; Ferguson and Moccia, 1980; Bassleer, 1983; Post, 1987; Andrews *et al.*, 1988; Gratzek, 1988; Paull and Matthews, 2001). In ornamental fish, these flagellates have been associated with systemic infections, with the "*hole in the head*" disease (Paull and Matthews, 2001), and commonly found in a wide variety of hosts (Poynton and Sterud, 2002). This study reports a diplomonad flagellates infection in some ornamental fish in the family cichlidae and belontidae in Thailand. The parasite prevalence, ultrastructure, pathogenicity, susceptibility of different fish species as well as the treatment of spironucleosis are reported.

2. Materials and Methods

2.1 Fish

Between November 2008 and May 2009, 121 angelfish (*P. scalare*), 100 blue mbuna (*L. fuelleborni*), 110 oscar (*A. ocellatus*), and 98 Siamese fighting fish (*B. splendens*) with an average weight of 2.46±0.30, 3.17±1.11, 5.56±1.16 and 3.19±0.31g, respectively, were collected from commercial ornamental fish farms, Bangkok and Ratchaburi Province and transported alive to the Kidchakan Supamattaya Aquatic Animal Health Research Center, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Each fish species was kept separately in glass aquaria to prevent cross infections. Water temperature during maintenance was 26-28°C and 20% water exchange was carried out for each system every two days. Test fish were fed daily with commercial feed at 3-5% body weight.

2.2 Detection of diplomonad flagellates

Infected fish were anesthetized with 50 ppm quinaldine (Coyle *et al.*, 2004) and recorded for clinical signs. Internal organs including intestine, spleen, heart, and stomach were dissected and smeared onto a glass slide. Unstained flagellates were inactivated with 5% formalin prior to examination and measurement for body size under light microscope. Internal organs were also imprinted on glass slides and stained with diff quick (Baxter Diagnostics) to allow characterization of the parasite morphology and nuclei shape. Prevalence of diplomonad flagellate in each ornamental fish is calculated as percentage from the number of infected fish to the total number of fish examined.

2.3 *In vitro* culture of diplomonad flagellates

Intestine of infected angelfish, blue mbuna and oscar were dissected aseptically, minced and inoculated in 10 ml of Leibovitz's L-15 medium supplemented with 3% fetal bovine serum, 1% heparin, 10 µg/ml of penicillin-streptomycin, 150 µg/ml of penicillin G, 150 µg/ml of streptomycin, 150 µg/ml of gentamicin, 40 µg/ml of amphotericin B, 10 µg/ml of bovine bile (Sigma) and 0.01 g fresh tilapia liver. Cultures were incubated at 25°C and viable cells were observed daily with an inverted microscope.

2.4 Electron microscopy

Diplomonad flagellates, grown in culture medium as described above, were harvested by centrifugation at 1,500 rpm for 5 minutes and re–suspended in fresh Leibovitz's L-15 medium. A suspension of parasite was smeared on a gelatin coated slide and fixed with 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) at 4 °C for 1 hr, then post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4). The sample was then dehydrated through a graded ethanol series and dried under a critical point dryer (CPD-Hitachi), then coated with 15 nm gold particles in a Polaron sputter coater. Parasites were viewed with a Jeol scanning electron microscope (SEM) at 15 kV and images recorded digitally for later printing.

For transmission electron microscope (TEM), samples were fixed as described for SEM, and then post-fixed with 1% OsO_4 in Veronal acetate buffer (pH 7.3). Samples were embedded in Epon-812 and thin sections stained with uranyl acetate and lead citrate. They were viewed with a Zeiss EM 109 at 80 kV.

2.5 Identification to genus and species

Parasites were identified to the genus and species based on parasite morphology under light and electron microscopy using the descriptions of diplomonad flagellates isolated from fish as described by Poynton and Sterud (2002) and Poynton *et al*. (2004).

2.6 Pathogenicity study

Angelfish with an average weight of 1.81±0.44 g were maintained in aerated glass aquaria and acclimatized for two weeks before infectivity testing. During the acclimatization period, these fish were fed daily with commercial feed at 3-5% body weight. The fish were examined to ensure that they were disease-free prior to use in the trial. They were anaesthetized with 50 ppm quinaldine and injected intraperitoneally with 0.1 ml of a parasitic suspension. Briefly, the parasites grown in culture medium were harvested and re–suspended in phosphate buffer saline (pH 7.4) to achieved final concentration of $1.1x10^3$, $4.2x10^3$, $1.2x10^4$, $4.6x10^4$, $1.12x10^5$, $4.5x10^5$ and $1.25x10^6$ cells/ml. A control group was injected with sterile PBS (pH 7.4). The experiment was conducted in triplicate using 10 fish per replicate. Water temperature during the assay was 26-28°C. Clinical signs and

mortality were recorded daily for 14 days post challenge. Tissue samples of intestine, stomach, liver, spleen and heart were aseptically collected and examined for diplomonad flagellates. The mean 14–day-LD_{50} was determined using the simplified method of Reed and Muench (1938).

2.7 Histopathology

Tissue samples of intestine, liver, stomach, gall bladder, heart and spleen from diseased fish obtained from both commercial fish farms and experimentally infected conditions were fixed in 10% buffered formalin solution and processed using standard histological techniques (Humason, 1979). Briefly, after 72 hrs fixation, each sample was cut into small pieces for better penetration of the reagents in the process of paraffin embedding. Respective tissue samples were placed in tissue cassettes labeled with corresponding source of samples. Histological sections were processed routinely, sectioned at 3-5 μm, and stained with haematoxylin and eosin (H&E). The stained sections were examined under a light microscope.

2.8 Susceptibility of other fish species

Thirty goldfish (*Carassius auratus*) with an average weight of 5.94±1.88 g, thirty guppy (*Poecilia reticulata*) with an average weight of 0.81±0.24 g and thirty platy (*Xiphophorus maculatus*) with an average weight of 0.44± 0.21 g were inoculated intraperitoneally with 0.1 ml of $3.0x10^3$ cells of diplomonad flagellates prepared as described above. Ten control fish from each species were injected with 0.1 ml PBS (pH 7.4). Observation was made daily over a period of 10 days for signs of disease. Tissue samples from all dead or survival fish were aseptically collected and examined to confirm diplomonad flagellates as the cause of death.

2.9 Growth inhibition assay

Dimetridazole, metronidazole and magnesium sulfate ($MgSO_4$) were used to inhibit the growth of diplomonad flagellates *in vitro*. Dimetridazole and metronidazole were examined at concentrations of 0.25, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0, and 10 μg/ml by dissolving in dimethyl sulfoxide. $MgSO_4$ was examined at concentration of 2.5, 5.0,10, 20, 40, 60, 80, and 100 mg/ml by dissolving in distilled water. Stationary phase of diplomonad flagellates grown in culture medium at concentration of $2.5x10^3$ cells/ml were used in the study. Control groups were established for each chemotherapeutic testing group. All treatments were examined in triplicate in sterile screw-capped test tubes containing 5 ml diplomonad flagellates, which were incubated at 25ºC in a thermo-regulated incubator. The average number of parasites was determined every 24 hrs until no parasites was observed alive (Sangmaneedet and Smith, 1999).

2.10 *In vivo* treatment of spironucleosis in angelfish

Dimetridazole was chosen for treatment of spironucleosis in angelfish because of its effectiveness in inhibiting parasite growth *in vitro* study. Infected angelfish collected from ornamental fish farm were used in this study. Infected fish were sampled and determined for the present of the diplomonad flagellates using the same method as described previously. This experiment was conducted in duplicate using 45 fish per replication. Dimetridazole was examined at concentrations of 0, 0.25, 0.50, 1.0, 2.0, 4.0, and 6.0 μg/ml by prolonged immersion method. After immersion, five infected fish were collected daily from each replication to examine the present of the parasite. Infection rate was calculated as percentage from number of infected fish to the total number of fish examined.

3. Results

3.1 Clinical signs

The study of diplomonad flagellates infection revealed the infection in 3 out of 4 ornamental fish species i.e., angelfish, oscar and blue mbuna. The highest percentage of infection was recorded in angelfish followed by oscar and blue mbuna (Table 1). Infected fish showed various disease signs including lethargy, emaciation, anorexia, abdominal edema and fecal pseudocasts, pale skin with fin and tail rot (Figure 1). The infection was found in several organs of infected fish including intestine, liver, gall bladder, spleen, heart, skin and fins. No diplomonad flagellates was observed in Siamese fighting fish.

3.2 *In vitro* culture of diplomonad flagellates

Diplomonad flagellates from the angelfish intestine were successfully established and maintained in culture media. The cultured cells reached maximum numbers within three days post inoculation. The parasite died within five days if sub-culture was not made (Figure 2). No multiplication of the parasites isolated from blue mbuna and oscar was observed.

3.3 Identification of diplomonad flagellates

Morphological characteristics of diplomonad flagellates from angelfish under light and electron microscopes studies indicated that the parasite was *Spironucleus vortens*. In squash preparations, parasites were highly motile and rotated around their longitudinal axis. Trophozoites were pyriform to ovoid in shape, 9.0-16.0 μm (mean 12.86 μm) in length and 3.0-10.0 μm (mean 6.52 μm) in width. The parasite had distinct S-shaped nuclei and eight flagella (six anterior and two posterior).

Table 1. Prevalence of diplomonad flagellates in ornamental fish cultured in Thailand.

Host	Prevalence (%) (infected fish/total fish)	Organ of infection
Angelfish	90 (109/121)	Skin, fin, intestine, liver, stomach, heart, gall bladder and spleen
Oscar	75.4 (83/110)	Skin, fin, intestine, liver, stomach, heart, gall bladder and spleen
Blue mbuna	61 (61/100)	Skin, intestine, liver, stomach, heart, gall bladder and spleen
Siamese fighting fish	0 (0/98)	-

SEM of trophozoites gave slightly smaller dimensions than fresh material with mean length of 10.5 µm (8.5-12.5 µm) and mean width of 3.05 µm (2.5-4.6 µm). The six anterior flagella emerged posterior-medially from the cytostome opening. Two recurrent flagella protruded from the posterior end of the body surrounded by a crescent-shaped ridge. Parasites had an adorned body surface with compound lateral ridges counter crossing at posterior end with papillae (Figure 3).

TEM revealed that the parasites consisted of a highly vacuolated cell with prominent recurrent flagella. Unique characteristics of the present diplomonad flagellate include kinetosomes just below the apex of the S-shape nuclei, and recurrent flagella with flagellar pockets (cytosomal canals) passing posteriorly through the cell (Figure 4).

3.4 Pathogenicity study

Infection of angelfish by intraperitoneally injection with *S. vortens* caused 13.33-86.67% mortality within 14 days (Figure 5). Most mortality occurred within three days post infection. Experimental fish infected with *S. vortens* showed clinical signs similar to natural infected fish, but some fish died without any clinical sign. Moribund fish were lethargic, swam near the surface, spun to the bottom of the aquarium and finally died. Characteristics of clinical signs included a depigmented skin, abdominal edema and fecal pseudocasts. The infection was found in several organs of infected fish including intestine, liver, gall bladder, spleen and heart. No mortalitiy occurred in the control group injected with sterile PBS pH 7.4. Pathogenicity study of *S. vortens* in angel fish showed 14–days LD_{50} of $2.99x10^3$ cells.

3.5 Histopathological changes

Histopathological changes of infected angelfish revealed granulomatous liver, infiltration of macrophage centers in the spleen and lymphocytic infiltration and inflammation of the intestinal submucosa (Figure 6-8). No histopathological changes were observed in the liver, spleen and

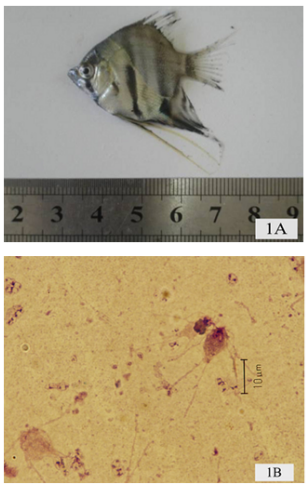

Figure 1. Gross photographs of angelfish (1A) severely infected with diplomonad flagellates showing excess mucus production, pale skin with fin and tail rot. Trophozoites of diplomonad flagellates from angelfish (1B) showing 2 nuclei with 6 anterior flagella and 2 posterior flagella (Diff quick, scale bar = 10 µm).

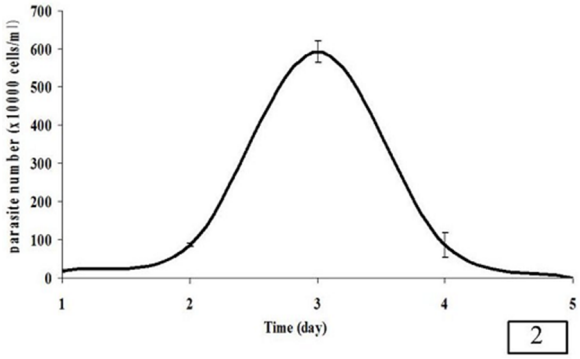

Figure 2. Growth of diplomonad flagellates from angelfish intestine in Leibovitz's L-15 medium containing 3% FBS, 1% heparin, 10 µg/ml penicillin-streptomycin, 150 µg/ml penicillin, 150 µg/ml streptomycin, 150 µg/ml gentamicin, 40 µg/ml amphotericin B and fresh tilapia liver.

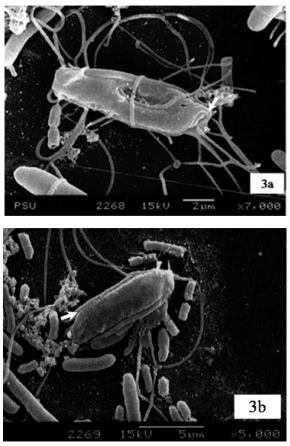

Figure 3. Scanning electron microscopy of *S. vortens* from angelfish showing a cresent-shape ridge (arrow) with papillae (3A). An adorned body surface with compound lateral ridges (arrow) counter-crossing at posterior end (3B). p = papillae.

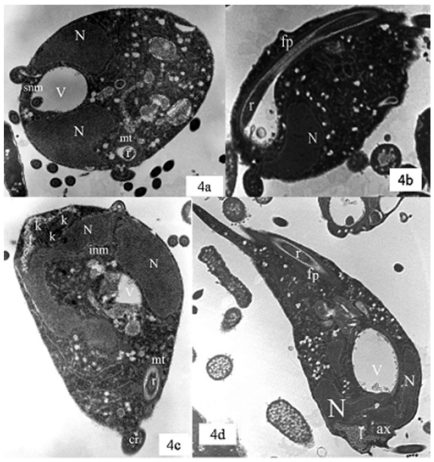

Figure 4. Transmission electron microscopy of *S. vortens* from angelfish showing an ovoid shaped with 2 S-shape nuclei and highly vacuolated cell (4A-4D). N = nucleus; k = kinetosome; V = vacuole; r = recurrent flagella; fp = flagellar pockets; snm = supra nuclear microtubular; inm = infra nuclear microtubular; cr =central ridge; mt = microtubule; ax = axoneme of anterior flagella; l = lamella.

intestine of oscar and blue mbuna infected with diplomonad flagellates.

3.6 Susceptibility of other fish species

No mortality or sign of disease was observed in goldfish, guppy and platy fish injected with *S. vortens* or any of the control fish. Although lethargy of guppy and platy injected with *S.vortens* was recorded, all fish recovered within one day post injection. No *S. vortens* was detected in survived fish examined at the end of experiment.

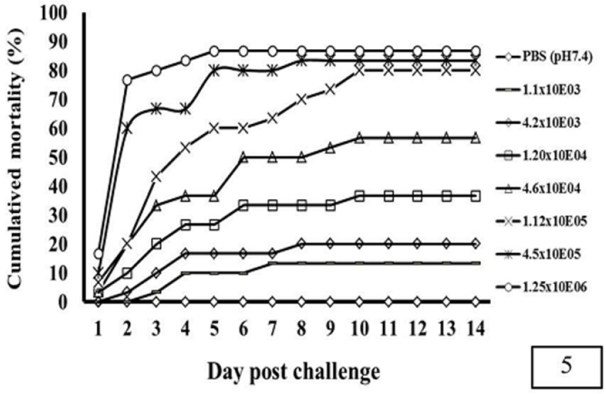

Figure 5. Cumulative mortality of angelfish intraperitoneally injected with various concentrations of *S. vortens*.

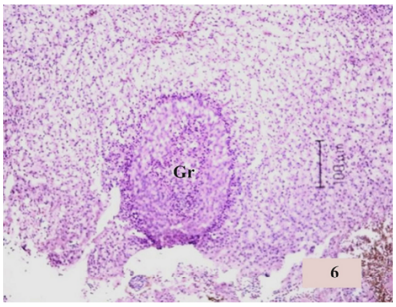

Figure 6. Liver tissue of angelfish infected with *S. vortens* showing granuloma formation (Gr) (H&E, scale-bar = 100 µm).

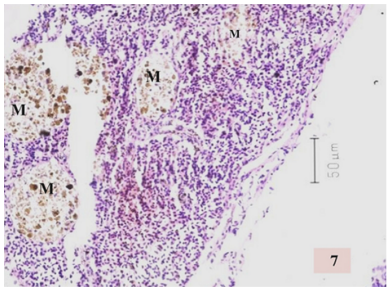

Figure 7. Spleen tissue of angelfish infected with *S. vortens* showing infiltration of macrophage center (M) (H&E, scale bar = 50 µm).

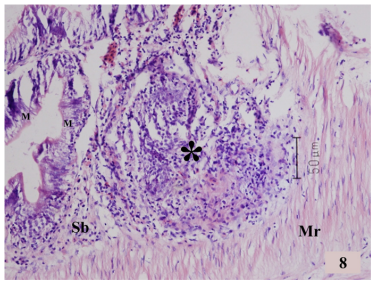

Figure 8. Intestine tissue of angelfish infected with *S. vortens* showing inflammation (*) and lymphocytic infiltration of intestinal submucosa. H&E; scale bar = 50 µm; M = mucosa; Sb = submucosa; Mr = muscularis.

3.7 Growth inhibition assay

In vitro inhibition assay revealed that 100% of *S. vortens* were killed after 48 hrs exposure to dimetridazole and metronidazole at concentrations of ≥4 µg/ml and ≥6 µg/ml, respectively (Figure 9 and 10). $MgSO_4$ inhibited the growth of *S. vortens* at a concentration of ≥60 mg/ml after 72 hrs exposure (Figure 11).

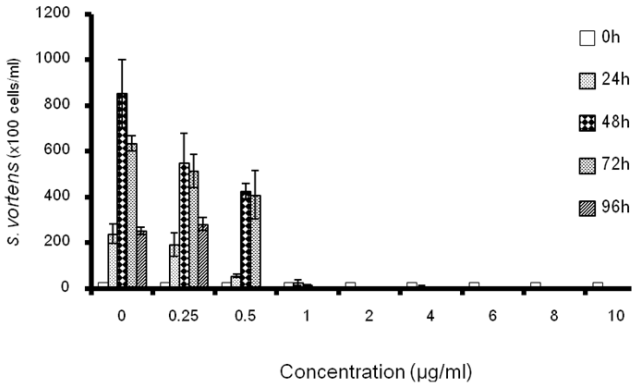

Figure 9. Growth of *S. vortens* in different concentrations of dimetridazole (µg/ml).

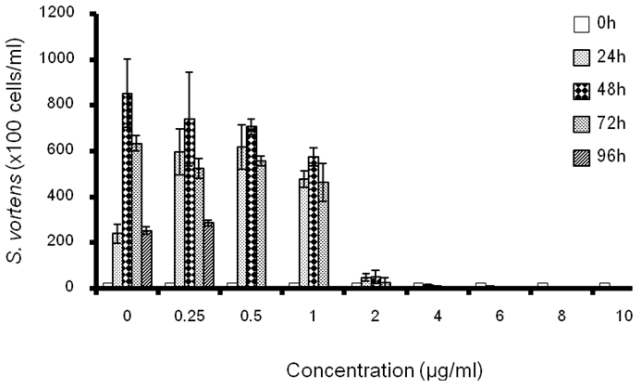

Figure 10. Growth of *S. vortens* in different concentrations of metronidazole (µg/ml).

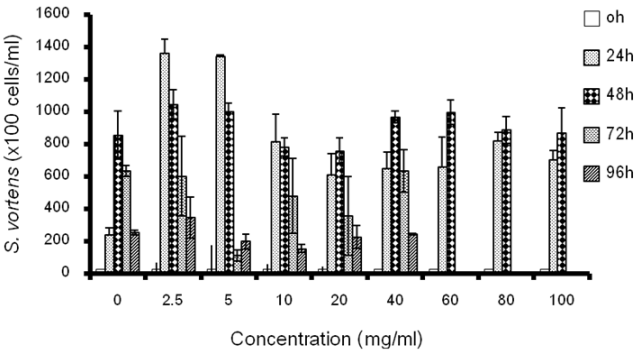

Figure 11. Growth of *S. vortens* in different concentrations of $MgSO_4$ (mg/ml).

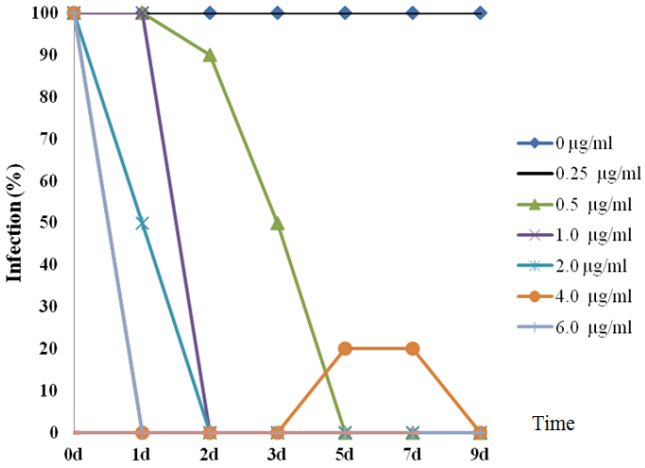

Figure 12. Efficacy of dimetridazole in inhibition of *S. vortens* growth in angelfish

3.8 *In vivo* treatment of spironucleosis in angelfish

In vivo treatment of spironucleosis in angelfish with dimetridazole revealed that *S. vortens* were killed after one day exposure at concentrations of 4 and 6 µg/ml. Dimetridazole at a concentration of 1 and 2 µg/ml killed the parasite after two days exposure, while a concentration of 0.5 µg/ml killed the parasite after five days exposure. *S. vortens* were observed alive using dimetridazole at a concentration of 0.25 µg/ml (Figure 12).

4. Discussions

Diplomonad flagellates infection in fish causes serious damage to aquaculture worldwide. Heavy infections of these parasites may interfere with normal growth of the host by competition with nutrient absorption (Yasutake *et al*., 1961). In the present study, various disease signs were observed in angelfish, blue mbuna and oscar infected with diplomonad flagellates. The infected fishes showed varied clinical signs from no (Mo *et al*., 1990) to severe symptoms (Kent *et al*., 1992). Infected Atlantic salmon exhibited anemia, skin blisters, muscle ulcerations, unilateral exophthalmia, hemorrhaging of internal organs, splenomegaly or deformed spleen, or granulomatous lesion in the spleen and liver (Guo and Woo, 2004). However, infected angelfish showed no symptoms of the disease although they were infected by *S. elegans* (Kulda and Lom, 1964). Diplomonad flagellate infections in fish encompass a wide geographical range from tropical to cold water area, from farm fish to wild fish and various hosts, which include fresh water and marine fish (Ferguson, 1979; Poynton and Morrison, 1990; Kent *et al.*, 1992; Poynton *et al.*, 1995; Sterud, 1998a, b). Only the genus *Spironucleus* infects fish, however few species of *Octomitus* or *Hexamita* sp. were found in fish (Paull and Matthews, 2001; Poynton and Sterud, 2002).

The present study successfully cultured diplomonad flagellates from angelfish, but failed to cultured diplomonad flagellates from blue mbuna and oscar although several attempts have been made. This may be explained by species difference and that the parasite may require some other nutrients for growth in culture media. However, identification on the basis of morphological characteristics indicated that the diplomonad flagellates isolated from angelfish was *S. vortens*. This parasite, an aerotolerant anaerobe that can be cultured at 25-30°C, was first described by Poynton *et al.* (1995). The parasite was isolated from the kidney, liver, spleen and head lesions of discus and also recorded from the head lesions of the angelfish (Paull and Matthews, 2001). In this study, no mortality was observed in goldfish, guppy and platy fish injected with *S. vortens* indicating that these experimental fish were resistant to artificial infection. Histopathology of fish infected with diplomonad flagellates varies from severe pathological changes to no obvious histological effect (Uzmann *et al.*, 1965). In this study, histopathological findings indicated systemic infections that could be found in several organs including liver, spleen and intestine. Similar results were recorded in Chinook salmon and Atlantic salmon infected by *H. salmonis* (Kent *et al.*, 1992; Poppe *et al.*, 1992).

Dimetridazole, metronidazole and $MgSO_4$ have been used in the *in vitro* treatment of spironucleosis in ornamental fish (Sangmaneedet and Smith, 1999). The present study indicated that dimetridazole is the most effective chemotherapeutic agent in inhibiting the growth of *S. vortens in vitro*. Moreover, the *in vivo* study revealed that dimetridazole at 4.0 µg/ml provides the highest efficiency, which can be used for treatment of spironucleosis in angelfish culture in Thailand.

In summary, the present study shows that diplomonad flagellates have a great impact on ornamental fish, especially angelfish, cultured in Thailand. Further investigation on the diplomonad flagellates isolated from blue mbuna and oscar may provide more insight into understanding the diplomonad flagellaes infection in ornamental fish cultured in Thailand.

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