SPIRONUCLEUS VORTENS OF THE FRESHWATER ANGELFISH (*PTEROPHYLLUM SCALARE*): GROWTH REQUIREMENTS, CHEMOTHERAPEUTANTS, PATHOGENESIS AND IMMUNITY

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

In

Veterinary Medical Sciences

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December 2, 1999

Blacksburg, Virginia

Keywords: Angelfish, Diplomonad, *Spironucleus vortens*, Chemotherapy, Parasite

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Abstract

For many years hexamitids, *Hexamita spp*. and *Spironucleus spp.*, have frequently been reported in vertebrates, particularly in fish. This suggests a potentially important role of these parasites in the fish culture industry. Though the majority of hexamitids are not known to cause disease in their vertebrate host, those that have been documented as associated with disease are still in need of further investigation into their geographical distribution, host range, life cycle, host-parasite relationship, pathogenicity, diagnosis, prevention, treatment, and control.

Spironucleus vortens is a hexamitid recently described from angelfish (*Pterophyllum scalare*). Although the structure of this parasite has been investigated using the electron microscope (Poynton *et al*., 1995), other information on this organism is poorly understood. Thus, the purpose of this research was to study the nature of *S. vortens* in TYI-S-33 culture medium and in the angelfish host. The optimal environmental conditions for *S. vortens* growth were investigated using variations of temperature, pH, and bile concentrations. This study is useful in helping to understand the locations and environmental conditions in the host that are suitable for the growth of *S. vortens*. Treatment of *S. vortens*, using seven chemotherapeutic agents; dimetridazole, metronidazole,

pyrimethamine, albendazole, fenbendazole, mebendazole, and magnesium sulfate was evaluated. The pathogenicity of *S. vortens* in angelfish was investigated in fish experimentally inoculated with trophozoites. This study provided information to help understand the pathogenesis of the parasites in the host. Finally, to examine the protective defense mechanisms, the presence of anti-*S. vortens* antibodies in angelfish serum were evaluated along with the presence of immune cells (lymphocytes, macrophages, eosinophilic granular cells, neutrophils, and plasma cells) at invaded sites of the intestine and other internal organs in response to an experimental *Spironucleus vortens* infection. The results of this research provide information on this parasite's effect on the fish host which may be useful in understanding the nature of other hexamitids.

A few published reports have suggested the *in vitro* growth requirement of fish *Spironucleus* (Poynton *et al*., 1995; Sterud, 1998), but none have examined the optimal conditions required for growth and the pathogenicity of *S. vortens*. The first study was to examine the optimal requirements for the *in vitro* growth of the parasite. The organisms were cultivated in either an artificial medium (TYI-S-33) at different temperatures or various pH conditions, or in medium supplemented with different bile concentrations at 25°C. Criteria used to justify the optimal conditions were average cell number ml^{-1}, growth rate, survival time, and cell conditions (motility and morphology). The organisms survived longest at 22°C, and had the highest average cell number ml^{-1} at 25°, 28° and 31°C. At 25°C the parasites were highly active and survived up to 6 days. The organisms cultivated at pH 6.5, 7.0 and 7.5 yielded the highest average cell number ml^{-1} with survival periods up to 13-14 days. Most of the organisms cultivated at a pH lower than 6.0 or a pH higher than 7.5 were suppressed and killed within 5-6 days of cultivation. All cultures supplemented with

bovine or fish bile yielded lower maximal numbers of parasites than cultures with no bile. These results indicate that the optimal condition for the *in vitro* cultivation of *S. vortens* is 25°C and pH 6.5 to 7.5 without supplementation with bile.

In order to treat spironucleosis, the efficacy of various chemotherapeutic agents on the growth of *S. vortens* was examined *in vitro*. In this study nitroimidazoles and benzimidazoles, formerly reported as drugs of choice for the treatment of diplomonads, pyrimethamine and magnesium sulfate (Epsom salt) were evaluated at different concentrations on the growth of *S. vortens*. Dimetridazole and metronidazole were effective in inhibiting the parasite's growth at concentrations of 1 µg ml^{-1} or higher. Albendazole and fenbendazole suppressed the growth of parasites at concentrations of 1.0 µg ml^{-1} or higher after 24 h exposure. Mebendazole was the most effective agent of the benzimidazole group; and inhibited the parasite's growth at concentrations of 0.5 µg ml^{-1} or higher. Pyrimethamine at concentrations of 1-10 µg ml^{-1} failed to inhibit the parasite's growth. Magnesium sulfate inhibited the growth of the parasites only at high concentrations (70 mg ml^{-1} or higher) . This study indicates that dimetridazole, metronidazole and mebendazole are the most effective chemotherapeutic agents *in vitro* at inhibiting the growth of *S. vortens.*

To investigate the pathogenesis of spironucleosis, angelfish were orally (PO) or intraperitoneally (IP) inoculated with *S. vortens*. Control angelfish which were orally gavaged or intraperitoneally injected with PBS were in normal body condition and had no morbidity or mortality. Compared to the control angelfish, PO-infected angelfish were inappetent with no other clinical signs, while IP-infected angelfish showed clinical signs of inappetite, weakness, respiratory distress, and laying on their sides. Twenty percent of the IP-infected angelfish died within the first three weeks after infection. In PO-infected

angelfish, the organisms were located only in the intestinal lumen. In IP-infected angelfish, *S. vortens* were found in the blood, stomach, intestine, and other internal organs (spleen, gall bladder, and ovary). However, no parasites were observed within the intestinal mucosa of either PO- or IP-infected fish. Histopathologic examination of the intestines revealed mild to moderate multifocal enteritis in both PO- and IP-infected angelfish. The mucosal epithelium appeared undamaged although the parasite was closely located and appeared attached to the intestinal mucosa. The results suggest that *S. vortens* normally causes mild to moderate multifocal enteritis with no morbidity. However, the parasites can cause granulomatous inflammation in a wide variety of host tissues, and may be lethal if they enter the abdominal cavity and disseminate to other organs via the blood circulation.

Immunity, both cell mediated and humoral, against *S. vortens* was investigated in this study. Histopathologic examination revealed a response from inflammatory cells infiltrated and localized in the affected tissues. Macrophages, lymphocytes, and plasma cells were the most common cell types found in the internal organs. Macrophages were active in the affected tissues where the parasites lived *in situ*. However, *in vitro* studies indicated that there were no differences in a production of H_2O_2 or in phagocytosis between macrophages of control and infected angelfish regardless of inoculum dosage and administration route. A preliminary study of humoral antibody indicated that angelfish did not develop anti-*S. vortens* antibody after they were orally or intraperitoneally infected with either a low or a high number of the organisms. It is suggested that localized leucocyte response may be an important mechanism against *Spironucleus vortens* infection in angelfish.

This research has indicated some of the important environmental factors affecting the parasite's growth, and has provided some initial information on the pathogenicity of *S. vortens*. In addition, preliminary information on the host's protective immune systems,

humoral and cell-mediated immunity, against the parasite have been documented. The results from this research will be useful for aquaculture, particularly of tropical freshwater angelfish, and may help to provide an understanding of the biological roles of other hexamitids.

ACKNOWLEDGMENTS

I wish to thank all whom have helped support and contribute to this study. I would like to express my gratitude to my major advisor, Dr. Stephen A. Smith, for his support, patience, encouragement, and especially for his guidance through this study. I would like to thank all of my committee members for their input, encouragement and guidance. I thank Dr. John L. Robertson for helping and teaching me in interpretation of tissue sections, and I thank Dr. Thomas Caceci for help in clarify details of electron micrographs. I also want to express my appreciation to Dr. Sarah L. Poynton for her guidance and suggestion to this effort, and to Dr. David S. Lindsay for his guidance and providing some of the chemotherapeutics used in this research, and Dr. Michael G. Levy who served as an external examiner.

I acknowledge all those who have helped me in these projects: Dr. Steven D. Holladay (information of chemiluminescence analysis), Dr. Jeffrey C. Wolf (histopathological information), Dr. Terry Hrubec (laboratory techniques), Dr. Bob Gogal and Ms. Joan Kalnitsky (flow cytometry analysis), Ms. Ginny Viers and Ms. Kathy Lowe (electron microscopic techniques), Ms. Sandy Brown (accessibility to fish facility), and Korawuth Punareewattana and Amornchai Somjetlertcharoen (help in collecting samples).

I also wish to thank Dr. John C. Lee, Associate Dean of Research and Graduate Studies, and Dr. Ludeman A. Eng, department head of Biomedical Sciences and Pathobiology, for support of my Ph.D. program. Most of all I wish to thank the Royal Thai Government for giving me financial support during my study in the USA.

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CHAPTER 1

INTRODUCTION

1.1: Angelfish (*Pterophyllum sp*.)

Freshwater angelfish are among the most popular of all tropical aquarium fish due to varieties of strains and the ease of maintenance. Angelfish are indigenous to the Amazon River Basin in South America, and they have been successfully bred and raised as aquarium fish since the 1940s (Axelrod, 1985). The angelfish is classified in the Family Cichlidae; with a strongly laterally compressed, disc-shaped body and long, elegant fins. Fully grown angelfish are up to 5 inches in length and up to 8 inches in height. There are 4 nominal species of angelfish recognized at present; *Pterophyllum scalare, P. leopoldi*, *P. dumerilii* and *P. altum* (Sakurai et al., 1993).

Angelfish are considered one of the most economically important species to Florida state's ornamental fish growers (Specht et al., 1989), and its worldwide production has been estimated at 25 million fish per year (Axelrod, 1985). Since the discovery of the original black-striped silver angelfish, new types of angelfish have been produced such as *P. scalare* var. marble angelfish, *P. scalare* var. blushing angelfish, *P. scalare* var. golden angelfish, and *P. scalare* var. black angelfish. The price of angelfish vary with size, variety, and breed; prices increase substantially for the more exotic varieties (veil-tails, blushing) (Specht et al., 1989). With varieties of types and colors, the angelfish are normally raised in groups, and sometimes with other fish.

The recommended conditions for raising angelfish in aquarium are water temperature between 22-30°C (72-86°F) (Mills et al., 1988), pH 5.8-7.5, and general hardness of water between 4-18° dH (degree of German hardness) (Andrews and Baensch, 1991) with a minimum dissolved oxygen (DO) of 5.0 mg/L (Smith, 1994). Angelfish are sensitive to impurities in the water; for example, nitrite causes the tips of angelfish's fins to erode (Andrews and Baensch, 1991). There are various kinds of diet to feed the fish such as worms, crustaceans, insects, plant matter, and dried food (Mills et al., 1988). To maximize the fish's growth, angelfish should be fed at least twice a day or more with a small amount of feed.

There are few external characteristics to differentiate between male and female angelfish. The genital papilla in sexually mature fish is normally used to determine sex. At breeding time, the female's genital papilla (egg-depositing tube) extends from the vent and is broader and blunter than the male's (Mills et al., 1988). Angelfish exhibit typical cichlid spawning behavior; a pair will separate themselves from the others. Females will begin spawning in 1-2 days after cleaning egg-laying sites which may be plant leaves, slate, or other vertical surface. The female creeps along the site laying down the egg in a linear fashion with the male following and fertilizing them in the same fashion (Sakurai et al., 1993). Parents will then guard the eggs until the fry are free-swimming.

1.2: *Hexamita* and *Spironucleus*

Diplomonads are parasitic protozoa classified in the Order Diplomonadida (Phylum Sarcomastigophora). The typical character of members in this Order is having two karyomastigonts which are bilaterally symmetrical or diplozoic (Levine, 1985; Woo and

Poynton, 1995). Each side of cell has a nucleus at the anterior end, and four flagella of which one is recurrent and free at the pointed posterior end (Lee, 1985; Levine, 1973). Other ultrastructural characteristics that can be found in diplomonads are rough endoplasmic reticulum, digestive vacuoles, and rosettes of glycogen granules, however, some organelles such as mitochondria, Golgi apparatus, and microbodies are absent (Ferguson, 1979; Kulda and Nohynkova, 1978; Levine, 1985). The diplomonads are extracellular flagellates with direct life cycles and are normally found in the intestinal tract of vertebrates (Woo and Poynton, 1995). The trophozoite is the stage which becomes more spherical prior to multiplication by binary fission, and later forms a cyst which is oval in shape. Infection from one host to another normally occurs after ingestion of the trophozoites or cysts passed into the environment with feces (Woo and Poynton, 1995). Although diplomonads have been reported in vertebrates, many of them are not known to cause disease while in others their pathogenicity is poorly understood.

Diplomonad flagellates, belonging to the genera *Hexamita* and *Spironucleus*, have been reported in the digestive tract of both freshwater and saltwater fish (Ferguson, 1979; Kulda and Lom, 1964a; Mo et al., 1990). These diplomonads have direct life cycles and multiply by longitudinal binary fission. They are closely related and are collectively called diplomonads or hexamitids. They are considered the pathogens causing hexamitiasis, spironucleosis and possibly hole-in-the-head disease in salmonids, cyprinids and ornamental tropical aquarium fish (Woo and Poynton, 1995). *Hexamita sp.* has been reported in coldwater fishes, while *Spironucleus sp.* has been reported in warmwater aquarium fishes such as angelfish (Kulda and Lom, 1964b; Poynton et al., 1995), cichlids including juvenile *Cichlasoma severum* (Bassleer, 1983), and in coldwater Atlantic salmon *Salmo salar* (Sterud et al., 1997).

1.2.1: Morphology

The morphology of the trophozoite stage of diplomonads varies from spherical to elongate. The body of *Hexamita* and *Spironucleus* is transparent and lacking in detail in living form. Some ultrastructural features used to distinguish between trophozoites of the genera *Hexamita* and genera *Spironucleus* are position and shape of nuclei, position of kinetosomal complexes, and depth of kinetosomal pocket in nucleus (Kulda and Lom, 1964a; Woo and Poynton, 1995). *Hexamita* may measure up to 18 μm long and 10 μm wide (Becker, 1977). The two opposed nuclei of *Hexamita* are oval or spherical in shape and located at their flattened medial portions. There are two opposed kinetosomal complexes located anterior-lateral of each nucleus, and the depth of the kinetosomal pockets in the nucleus of *Hexamita* is shallower than those in *Spironucleus* (Woo and Poynton, 1995). The two opposed compact nuclei of *Spironucleus* are S-shaped and wrap around each other at their narrow anterior ends (Kulda and Lom, 1964a). There are also two opposed kinetosomal complexes found in *Spironucleus*, and they are located at the anterior-medial portion (Woo and Poynton, 1995). Some other ultrastructural features that may be used to differentiate species of Diplomonadida are papillae, surface tori, lateral compound ridges, cytoskeleton, and microfilaments (Ferguson, 1979; Poynton and Morrison, 1990).

Summary of ultrastructural features between trophozoite of *Hexamita* and *Spironucleus*

Ultrastructural features	*Hexamita*	*Spironucleus*
Shape of nuclei	Oval or Spherical	S-shaped
Position where nuclei apposed	Median	Anterior end
Position of kinetosomal complexes	Anterior-lateral	Anterior-medial
Depth of kinetosomal pocket	Shallow	Deep

Little is known about the host specificity of hexamitids in fish because their morphological identifications have been evaluated only using a light microscope. Many investigations of hexamitids reported in fish have been based on the morphology of their bodies and nuclei, and most of them were reported as *Hexamita salmonis*. However, these criteria are unreliable to distinguish the two genera because the shapes of the body and nuclei may be variable in light microscope preparations (Poynton and Morrison, 1990). Sterud et al. (1997) suggested that *Hexamita* in fish has been inadequately studied by transmission electron microscopy, therefore it is possible that all fish diplomonads are in fact *Spironucleus*. Only four hexamitids from fish, *H. salmonis* (Ferguson, 1979), *S. torosa* (Poynton and Morrison, 1990; Sterud, 1998), *S. vortens* (Poynton et al., 1995), and *S. barkhanus* (Sterud et al., 1997) have been studied and confirmed by ultrastructure investigations. *Spironucleus vortens* found in angelfish are pyriform with a body 12.5–20.5 μm long and 5.0–11.2 μm wide. The trophozoite has 2 compound lateral longitudinal ridges. Each ridge originates anteriorly, close to the three emerging anterior flagella, and then continues posteriorly to the emergence of posterior flagellum (Poynton et al., 1995). There are two papillae at the posterior end of *S. vortens*' body (Poynton et al., 1995) while *S. torosa* have a caudal projection (tori) at the posterior end of their body (Poynton and Morrison, 1990; Sterud, 1998).

1.2.2: Host Range and Geographical Distribution

There are six species of *Hexamita* described from fish (Lom and Dyková, 1992). *Hexamita salmonis*, the causative agent of hexamitiasis, has been reported commonly in trout and salmon hatcheries throughout North America (McElwain, 1968; Yasutake et al., 1961) and in many other countries (Mo et al., 1990). *Hexamita salmonis* were found in greatest numbers in the upper intestine and pyloric region, and has been reported to cause ascitis, enteritis, and anemia in salmonid fish (Ferguson, 1979; Ferguson and Moccia, 1980; Uzmann et al., 1965; Woo and Poynton, 1995). They are also found in large numbers in the abdominal cavity, mesentery and the parenchyma of the liver, spleen and kidney (Ferguson and Moccia, 1980). They have also caused disease in Siamese fighting fish (*Betta splendens*) (Ferguson and Moccia, 1980) and high mortality in seawater chinook salmon in Canada (Kent et al., 1992). Transmission of *H. salmonis* may occur by cohabitation with infected fishes, by intraperitoneal injection of infected ascitis fluid, by gavage of infected ascitis, and by waterborne exposure with a mixture of infected ascitis and tissue (Kent et al., 1992). Although *Hexamita* have been mainly reported in fish, they can also be found as pathogenic organisms in turkeys (*H. meleagridis*) and the intestinal tracts of laboratory animals such as rats and mice (Wagner et al., 1974). Thus, there is some suggestion that fish may also harbor the parasite in a subclinical state.

In freshwater fish, *Spironucleus sp.* has been reported in cichlids, including angelfish, and cyprinids. *Spironucleus elegans* has been found causing disease in angelfish (*Pterophyllum scalare*) in Europe (Kulda and Lom, 1964b), while *Spironucleus vortens* has been also reported from the intestinal lumen of angelfish bred in Florida (Poynton et al., 1995). They can be found in the intestinal tracts of healthy fish and they may invade the body of the host and are capable of causing severe parasitemia under stress conditions (Molnár, 1974).

The predilection site of *Spironucleus* is in the posterior two-thirds of the intestine of cyprinids and aquarium fishes (Kulda and Lom, 1964a; Molnár, 1974). In grass carp (*Ctenopharyngodon idella*), they have been found in the posterior two-thirds of the gut, and they have also been found abundantly in muscle taken from the affected tissues of angelfish (Molnár, 1974). Both *S. elegans* (Kulda and Lom, 1964b) and *S. vortens* (Poynton et al., 1995) have been reported in the posterior intestine of *Pterophyllum scalare*. An incidence of spironucleosis in marine fish was reported in 1990 (Poynton and Morrison, 1990). These flagellates, *Spironucleus torosa,* were found from the rectum of the marine gadoids, Atlantic cod *Gadus morhua* L., and haddock *Melanogrammus aeglefinus. Spironucleus torosa* have been also reported in saithe (Sterud, 1998). Recently, a new hexamitid flagellate, *Spironucleus barkhanus*, has been reported from the intestine and gall bladder of wild grayling *Thymallus thymallus,* and from muscle abscesses of farmed Atlantic salmon, *Salmo salar* (Sterud et al., 1997). This parasite was formerly reported as a *Hexamita*-like organism causing disease in fingerlings and postmolt Atlantic salmon in Norway (Poppe et al., 1992).

Summary of hexamitids reported in fish.

Hexamitids	Host	Host Location	Geographical Location	Reference
Octomitus (*Hexamita*) *salmonis*	*Salmo shasta*, *S. leverensis*, *S. fario*, *Salvelinus fontinalis*, *Cristivomer namaycush*	intestine	New York, USA	Moore, 1922
H. salmonis	*Oncorhynchus tshawytscha*	intestine	Washington, USA	Yasutake, 1961
H. salmonis	*O. kisutch*, *O. tshawytscha*	caecum	Washington, USA	Uzmann, 1963
H. salmonis	*S. gairdneri*	intestine	Michigan, USA	Allison, 1963
H. salmonis	*O. kisutch*, *S. gairdneri*	intestine pyloric caeca	Washington, USA	Uzmann, 1965
H. salmonis	*S. gairdneri*	pyloric caeca	New Mexico, USA	McElwain, 1968
H. salmonis	*S. gairdneri*	intestine	Japan	Sano, 1970
H. salmonis	*S. gairdneri*	pyloric caeca, upper intestine	Northern Ireland	Ferguson, 1979
H. salmonis	*S. trutta*, *S. gairdneri*	pylorus, bile, mucus from skin and fin	UK	Poynton, 1986
H. salmonis	*O. tshawytscha*	blood, liver, kidney, spleen, pancreas, heart, brain, gills, lower intestine, mucus	British Columbia, Canada	Kent et al., 1992
H. salmonis	*O. mykiss*	intestine, pyloric caeca	Denmark, Europe	Buchmann, 1995, 1996
H. salmonis	*O. mykiss*	intestine	Spain, Europe	Tojo & Santamarina, 1998

Hexamitids	Host	Host Location	Geographical Location	Reference
H. salmonis	*Salvelinus fontinalis*	pyloric region	Nova Scotia	Poynton, 1990
hexamitid	*Betta splendens*	abdominal cavity, mesentery, stomach, blood vessels, liver, spleen, kidney	Canada	Ferguson, 1980
hexamitid	*P. scalare*	intestine	Florida, USA	Specht, 1989
Spironucleus sp.	*Ctenopharyngodon idella, Hypophthalmichthys nobilis, Cyprinus carpio, Chondrostoma nasus, Barbus barbus, Pterophyllum scalare*	Intestine, liver, muscle, gall bladder, visceral organs	Europe	Molnár, 1974
S. elegans	*P. scalare*	intestine	Czechoslovak, Europe	Kulda, 1964
S. torosa	*Gadus morhua, Melanogrammus aeglefinus*	rectum, pyloric caeca	Nova Scotia	Poynton, 1990
S. torosa	*Gadus morhua, Pollachius virens Lota Lota*	rectum	Southeastern Norway	Sterud, 1998
S. barkhanus	*S. salar*	heart, spleen, pyloric caeca, posterior gut, eye, brain	Northern Norway	Mo, 1990 Poppe, 1992
S. barkhanus	*Thymallus thymallus Salvelinus alpinus* *S. salar*	gut, gall bladder muscle abscesses	Norway	Sterud, 1997, 1998
Spironucleus sp.	*P. scalare*	intestine	Canada	O'Brien, 1993
S. vortens	*P. scalare*	intestine	Florida, USA	Poynton, 1995

1.2.3: *In Vitro* Cultivation and Maintenance

Cultivation of diplomonads has been attempted with various degrees of success. Axenic (pure) cultures are advantageous for biological and physiological investigations, since various host factors are not involved. The first report of an axenic culture of *Hexamita* species was by Uzmann and Hayduk in 1963. They isolated and cultured *H. salmonis* from silver salmon (*Oncorhynchus kisutch*) and chinook salmon (*O. tshawytscha*) in medium supplemented with human serum, lacalbumin hydrolysate, and antibiotics. They found that reduction of oxygen tension in fluid media with increasing nitrogen gas was necessary to maintain and increase the number of *H. salmonis*. *Hexamita salmonis* could survive in the environment at pH ranges from 5.5 to 9.0; with the pH 7.5 to 8.0 giving the highest propagation *in vitro* (Buchmann and Uldal, 1996). The life cycle of *H. salmonis* was observed *in vitro* by cultivation in minimum essential medium (MEM) supplemented with calf serum and antibiotics (Uldal, 1996). Their study provided a method to obtain trophozoites and cysts of *H. salmonis*. The trophozoites of these protozoa congregated in clusters and adhered to each other by their flagella before the encystment stage. Abundant cysts were found in cultures incubated at 10°C; and the spherical multiflagellated trophozoites were also found with the highest densities at the same condition. The optimal temperature for a population increase of this species was 10°C *in vitro*, although it had been shown to survive at 15°C and 20°C (Buchmann and Uldal, 1996). Another attempt to isolate and cultivate *Hexamita* was performed in shellfish (Khouw et al., 1968). *Hexamita inflata* were isolated and cultivated from the eastern oyster *Crassostrea virginica* after five serial passages in a beef serum or egg yolk enriched medium containing antibiotics. The organism, *H. inflata*, was maintained at 15–18°C with transfer to fresh medium at 7- to 10-day intervals (Khouw et al., 1968).

The first successful attempt at axenic culture of *Spironucleus* from fish was by Poynton *et al*. (1995). They isolated *Spironucleus vortens*, an aerotolerant anaerobe, from the intestinal tract and lip tumor of the freshwater angelfish (*Pterophyllum scalare*). The organism was cultivated in a modified TYM medium containing casein hydrolysate and bovine serum; the medium was also supplemented with penicillin and streptomycin (Poynton et al., 1995). *Spironucleus vortens* could be cultured and maintained at 25°C to 30°C. This isolate of *S. vortens* was also able to be cryopreserved in a cryoprotective agent, DMSO, and could be recovered in TYI-S-33 medium (trypticase, yeast extract, iron serum) with a recovery rate greater than 90% (Poynton et al., 1995). *Spironucleus barkhanus* has also been cultured and maintained in TYI-S-33 medium. However, this organism needs bile supplemented in the medium with incubation temperature at 5°C (Sterud et al., 1997).

TYI-S-33 medium has been shown to be very useful for the cultivation of diplomonad flagellates. It was first modified for the axenic cultivation of *Entamoeba histolytica* (Diamond et al., 1978). This medium was modified from TP-S-1-monophasic medium by replacing panmede, a papain digest of ox-liver, with yeast extract supplemented with iron, vitamin B_{12}, thioctic acid and Tween 80 (Diamond et al., 1978). The advantages of TYI-S-33 medium were less inocula was needed to maintain the cultures, more uniform morphology of yielded amoebae, and a small generation period when compared to the former medium (Diamond et al., 1978). The TYI-S-33 medium supplemented with bovine bile was also successful in axenic culture of four strains of *Giardia lamblia* (Keister, 1983). The numbers of cell yields per unit volume of *Giardia lamblia* cultured in TYI-S-33 medium was related to the ratio of culture vessel to medium volume (Keister, 1983).

1.3: Pathogenicity of Hexamitiasis and Spironucleosis

1.3.1: Intestinal Infection

Free flagellates of diplomonads are the most commonly encountered stage found in the intestinal lumen (Woo and Poynton, 1995). The preferred locations of trophozoites of *H. salmonis* are the anterior intestine and pyloric region (Moore, 1922). However, the parasite can be found throughout the entire length of the intestinal tract in a severe infection (Uldal and Buchmann, 1996). *Spironucleus vortens* normally occurs in the middle portion of intestine (Poynton et al., 1995) while *S. elegans* occurs in the posterior portion and rectum (Kulda and Lom, 1964b). *Spironucleus torosa* have been found in the rectum of marine gadoids (Poynton and Morrison, 1990). These organisms affect the morbidity and mortality of various salmonids, cyprinids and ornamental tropical aquarium fishes (Ferguson and Moccia, 1980; Poynton et al., 1995; Woo and Poynton, 1995). Transmission of the organism occurs via ingestion of either cysts or trophozoites, or when uninfected fishes co-habitate with infected fishes (Kent et al., 1992). Infections of hexamitiasis are easily transmitted during stressful events such as salmonid migration from freshwater to saltwater habitats (Kent et al., 1992). *Hexamita* are considered commensal organisms but can be pathogenic under various stressful conditions such as nutritional inadequacies, suboptimal water quality, crowding, poor sanitation and infections from other pathogens or parasites (Specht et al., 1989; Uzmann et al., 1965). Rainbow trout fry infected with *H. salmonis* showed no pathological changes in the epithelial cells of the pyloric caeca or upper intestine, and also no invasion of the intestinal surface found in these regions (Ferguson, 1979). However, the parasites have been reported causing catarrhal enteritis with excessive mucus production and the desquamation of epithelial cells in heavily infected fish (Sano, 1970;

Uldal and Buchmann, 1996). In severe infections, *H. salmonis* also caused reddening, hemorrhage and cellular damage in the intestine (Molnár, 1974; Yasutake et al., 1961).

The occurrence of spironucleosis is rare in common carp, while in angelfish and some other ornamental fishes the disease occurs frequently (Lom and Dyková, 1992). However, stressful conditions from long fasting and retention of chyme in the intestine induce multiplication of *Spironucleus* causing enteritis in grass carp (Molnár, 1974). In angelfish, *Spironucleus elegans*, a common intestinal flagellate of amphibians, were found in the lower intestine and the infections have been shown to be acquired experimentally by contact with amphibian fecal matter (Kulda and Lom, 1964b). Heavy infections of these diplomonad flagellates may interfere with normal growth of the host by competition with nutrient absorption (Yasutake et al., 1961).

Clinical signs of fishes infected by diplomonad flagellates vary from no clinical signs (Mo et al., 1990) to severe symptoms (Kent et al., 1992). Infected salmonids and aquarium fishes may show clinical signs of anorexia, anemia, emaciation, lethargy, hyperpigmentation, fecal pseudocasts, enteritis with excess mucus and yellow watery or jelly-like contents or swollen abdomen (Ferguson and Moccia, 1980; Kent et al., 1992; McElwain, 1968; Mo et al., 1990; Uzmann et al., 1965). *Hexamita salmonis*, in chronic infections, cause ulceration and perforation of the gastric mucosa in the Siamese fighting fish (Ferguson and Moccia, 1980). However, infected angelfish showed no symptoms of disease although they harbored abundant number of amphibian *Spironucleus elegans* in the posterior part of their intestines (Kulda and Lom, 1964a).

1.3.2: Systemic Infection

The pathogenicity of systemic hexamitid infections is poorly understood although it has been reported in salmonids (Kent et al., 1992; Poppe et al., 1992; Yasutake et al., 1961), cyprinids and aquarium fish (Molnár, 1974). It is believed that the organisms invade the intestinal epithelium and disseminate to other tissues when the host's resistance has been suppressed (Woo and Poynton, 1995). Histopathology of fishes infected from hexamitids varies from severe pathological changes to no obvious histological effect (Uzmann et al., 1965). *Hexamita salmonis* has never been reported to cause systemic infection in its host.

It has been suggested that only *Spironucleus spp.* cause systemic infection because they can invade intestinal mucosa and disseminate to other tissues (Siddall et al., 1992). Evidence of systemic spironucleosis was reported in cyprinids and aquarium fishes (Molnár, 1974), and in salmonids (Mo et al., 1990; Poppe et al., 1992; Sterud et al., 1997). In cyprinids, many *Spironucleus sp.* were found in the gut where they caused reddening of the mucous membrane, brownish-gray discoloration and necrosis of liver, and haemorrhagic enteritis. In aquarium fishes, the parasite has been reported to cause losses in angelfish stock of up to 50% of the population. The infected fishes showed reddening of the skin, and haemorrhages and ulcerations in the region of the head (Molnár, 1974). The parasites were found in the gut, gallbladder and visceral organs. *Spironucleus sp.* were also found in large numbers in the blood and the muscle of infected fish. In salmonids, *S. barkhanus* (formerly reported as *H. salmonis*-like organism) cause systemic infection in sea-caged Atlantic salmon *Salmo salar* (Mo et al., 1990; Poppe et al., 1992; Sterud et al., 1997). The parasites were found in many organs, including the liver, kidney, heart, spleen, pyloric caeca, posterior intestine, eye, and brain (Mo et al., 1990). Although systemic infection of *S. barkhanus* in

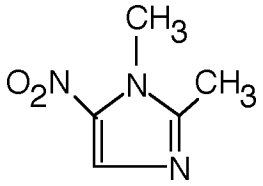
Atlantic salmon caused few external lesions, it caused granulomatous-like lesions containing large number of motile *S. barkhanus* in the mid- and posterior kidney and liver (Mo et al., 1990; Poppe et al., 1992). The liver and kidney were edematous, congested and haemorrhagic with multifocal necrosis. In severe cases, fish had extensive cholangiohepatitis, muscular degeneration, encephalitis and meningitis. Although *Hexamita* and *Spironucleus* cause disease in several economically important fish species in many regions of the world, their pathogenicity is not well known (Woo and Poynton, 1995). It has been believed that the effects and the severity of diplomonad infection are dependent on fish size, tissue site infested, environmental conditions, stocking density, season and stress factors (Allison, 1963; Buchmann and Uldal, 1996; Mo et al., 1990; Uldal and Buchmann, 1996). Fish infected with *Hexamita salmonis* exhibited clinical signs correlated to their body length and weight (Uldal and Buchmann, 1996). This supports the study of Allison (1963) that large trout, more than 3 or 4 inches long, showed a smaller effect from *Hexamita* infection than smaller trout. The incidence of disease is often related to a high stocking density of fry in hatcheries (Uldal and Buchmann, 1996), and occurs mainly during warm periods in the summer (Mo et al., 1990).

1.4: Chemotherapeutic Treatment

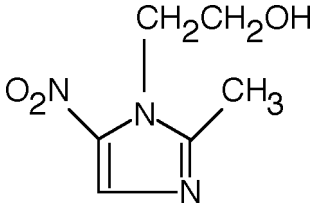
Heavy infections of diplomonad flagellates cause cellular damage in the intestinal tract of infected fishes, and the parasites may interfere with normal growth of the host (Yasutake et al., 1961). Treatment and control of these organisms are important in both the foodfish and tropical fish industry. Twenty-four chemotherapeutic compounds were tested in fingerling chinook salmon infected with *H. salmonis* (Yasutake et al., 1961). The effectiveness of chemicals in this testing was monitored by degree of infection, severity, relative weight gain

and fish mortality. Yasutake *et al*. (1961) found that at 0.2 percent in the diet, p-carbamidopheny arsenoxide, 2-amino-5-nitrothiazole, PR-3714 and fumagillin were effective in eliminating *H. salmonis* without toxic effects to the host. The efficacy of cyzine, 2-acetylamino-5-nitrothiazole, was also determined in trout infected with *H. salmonis* (McElwain, 1968). *In vitro*, cyzine at the concentrations of 0.20 and 0.08 ppm was effective in eliminating trophozoites within 24 hours; while *in vivo*, cyzine at 10 ppm was effective in controlling hexamitiasis in naturally infected rainbow trout (McElwain, 1968).

1.4.1: Nitroimidazoles



Dimetridazole



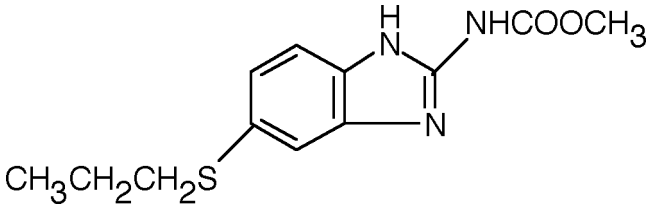
Metronidazole

In addition to the chemicals previously mentioned, other chemotherapeutic agents such as metronidazole, albendazole and mebendazole have been applied in the treatment of infections caused by diplomonads. Metronidazole, 1-(2-hydroxy ethyl)-2-methyl-5-nitroimidazole, is the effective compound of nitroimidazole used in the treatment of

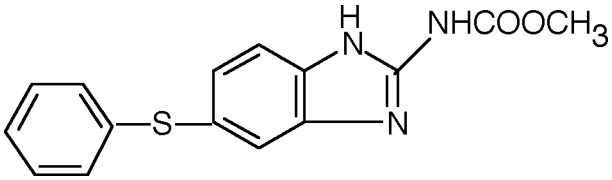
infections caused by anaerobic bacteria and protozoa (Amon et al., 1978; Baines, 1978; McLean et al., 1984). It has been applied frequently in the treatment of obligate anaerobic organisms having an active hydrogenase (Baines, 1978; Church et al., 1996). Metronidazole and related 5-nitroimidazoles are relatively nontoxic to the host organisms. They enter cells and are accumulated inside the cells by a passive diffusion method without an active transport process (Chapman et al., 1985; Müller, 1983). Their intermediate products, the reduced nitro-group forms, function as a short-lived cytotoxic effect, and the accumulations of these intermediates enhance the rate of entry of the drug into the susceptible cells (Edwards, 1980). Although the biologically active reduction products of metronidazole have not been isolated, it has been proposed that the toxicity of the intermediates is due to their interaction with DNA, decreasing the stability and integrity of DNA, and possibly with other macromolecules (Edwards, 1980; Müller, 1983). These radical anions generated within intact hydrogenosomes also may damage the radical-generating system and the hydrogenosome membrane, and are capable of efflux from isolated organelles (Chapman et al., 1985). At a low level (5.84 μM), metronidazole reduced the homogeneous material within the cytoplasm and caused the misshape of the trophozoites of *Spironucleus muris*; but did not affect the cytoskeleton (Oxberry et al., 1994). However, metronidazole has been shown to have very little effect upon the ultrastructure of *Giardia* (Oxberry et al., 1994), and the treatments were not successful in all cases. The fecal discharge of *Spironucleus muris* cysts in the mouse were decreased only when giving medicated drinking water (Kunstyr et al., 1977). Bassleer (1983) recommended metronidazole (Flagyl®) as an effective drug for the treatment of hexamitiasis and spironucleosis with a dosage of 500 mg/100 l of water for a 3-day period. Stoskopf (1988) recommended 10 mg of metronidazole mixed into 1.0 g of food given to fish for 5 days. Although metronidazole is the most widely prescribed drug in the treatment of anaerobic protozoa (Amon et al., 1978; Church et al., 1996; Edwards, 1980),

its unfavorable side effects in humans are nausea, headache, dark urine, high absorption, potential carcinogenicity, effect on normal bacterial flora and mutagenic effect on some bacteria (Edlind et al., 1990; Wolfe, 1982). In fish, an excessive use of metronidazole can damage kidneys and other internal organs (Bassleer, 1983).

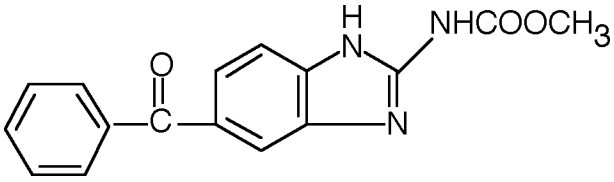
1.4.2: Benzimidazoles



Albendazole



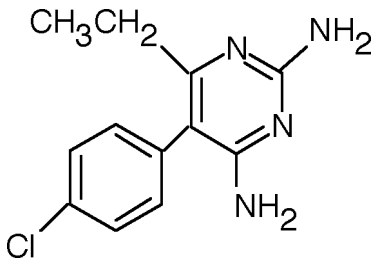
Fenbendazole



Mebendazole

Anthelmintic benzimidazole is another group that has been shown *in vitro* to effect intestinal protozoa such as *Giardia lamblia* (Bell et al., 1993; Edlind et al., 1990), *Giardia duodenalis, Trichomonas vaginalis* and *Spironucleus muris* (Oxberry et al., 1994). The target of benzimidazole in *Giardia sp.* is a structure called β-tubulin (Edlind et al., 1990) while antigiardial activity of bis-benzimidazole is its capability to bind DNA and ability to inhibit the activity of giardial topoisomerase II (Bell et al., 1993). With less absorbable, albendazole and mebendazole were 30 to 50 times more active than metronidazole (Edlind et al., 1990). Mebendazole inhibited growth of *Giardia lamblia* at a low concentration, 0.05 $\mu g\ ml^{-1}$, and killed *G. lamblia* at the concentration of 0.3 $\mu g\ ml^{-1}$ (Edlind et al., 1990). Mebendazole also has been shown to distort cell structures and rapidly disrupt the ventral disk and flagella of *G. lamblia* (Edlind et al., 1990), while albendazole binding to the cytoskeletal proteins causes trophozoites of *G. duodenalis* to become swollen, misshapen with cytoplasmic dark granular materials and finally die (Oxberry et al., 1994). In the treatment of *T. vaginalis*, albendazole increased glycogen granules and cytoplasmic vesicles and caused thickening of the parasite's cell coat with many invaginations (Oxberry et al., 1994). In contrast to the treatment of *Giardia* and *Trichomonas*, albendazole has no effect on the cytoskeleton or any other gross morphological characteristics of *S. muris* (Oxberry et al., 1994).

1.4.3: Pyrimethamine



Pyrimethamine

Pyrimethamine (5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidinediamine) was introduced for the treatment of malaria in 1952. It is also used in the prevention of relapsing encephalitis form of toxoplasmosis. Pyrimethamine is known as Darapram®, Daraprim®, Chloridin®, Malocide®, and Erbaprelina®. It works as an antifolate (dihydrofolate reductase inhibitor) on protozoa and bacteria. The organisms exposed to pyrimethamine cannot survive due to lack of tetrahydrofolate which is important in cell division (Catchpool, 1980). With the antifolate pharmacologic effect, pyrimethamine is frequently used in combination with other drugs such as sulfonamides or sulfones resulting in a synergistic antifolate potency.

1.4.4: Magnesium Sulfate (Epsom Salt)

Reducing the number of burden organisms by saline purges is an alternative method in the treatment of parasitic infestation, especially from luminal parasites. Saline purges all act in a similar mechanism by which the anions and cations are slowly absorbed from the digestive tract. Magnesium salts frequently used as saline purgatives are Magnesium Sulfate, (Epsom salts), Magnesium Hydroxide, Magnesium Oxide, (milk of magnesia), and Magnesium Citrate, (Jenkins, 1988). They retain or attract water into the intestinal lumen mainly by osmosis, which distends the gut and increases peristalsis, thus producing defecation. Another mode of action of magnesium salts is causing the release of cholecystokinin, which increases peristaltic activity of the intestine (Jenkins, 1988); intraluminal parasites then will be expelled from host. The concentration of saline purgatives should be isotonic such as 6% solution of Epsom salt to get a quick purgative action (Alexander, 1985). In the present study, magnesium sulfate which may be helpful in reducing the number of diplomonads was examined for an efficacy in inhibiting the parasite’s growth.

1.5: Fish Immunity

Fish immunity, humoral and cell-mediated immunity, has been shown to be an important defense system in disease resistance (Dickerson et al., 1989; Jones et al., 1993; Lin et al., 1996; Sharp et al., 1992; Woo, 1981; Yu et al., 1970). The host immune response can be demonstrated as the processes of phagocytosis (Avtalion and Shahrabani, 1975; Cross and Matthews, 1993), hemolysis (Sailendri and Muthukkaruppan, 1975), mitogen-induced lymphoproliferation (Wongtavatchai et al., 1995) or the appearances of immune complexes by agglutination, precipitation or viral neutralization (Clem, 1970). Numerous studies on piscine immunity have been reported in viral, bacterial and parasitic diseases of fish (Avtalion and Shahrabani, 1975; Piper et al., 1973; Plumb, 1973; Woo, 1992; Yu et al., 1970).

1.5.1: Humoral Immunity

The fish's humoral immune response has been studied in parasitic protozoan infections. Goldfish (*Carassius auratus*) infected with *Trypanosoma sp.* survived a re-infection after they developed immunity (Woo, 1981). The plasma of these goldfish also protected another group of goldfish from infection after the plasma was inoculated into them. There is also evidence that fish develop specific antibody against the dinoflagellate, *Amyloodinium ocellatum*. Tilapia (*Oreochromis aureus*) and sea bream (*Sparus aurata*) inoculated with *A. ocellatum* dinospores developed specific antibody which agglutinated and killed the parasite in cell culture (Smith et al., 1993). An anti-*Amyloodinium ocellatum* antibody was also produced in cultured hybrid striped bass (*Morona saxatilis* x *M. chrysops*). This antibody titer was higher in both infected fish in culture and the experimentally immunized fish than

in uninfected fish (Smith et al., 1994). The role of antibody against ciliated protozoan has been shown by Dickerson *et al*. (1989). They demonstrated that fish acquire immunity against the ciliated protozoon, *Icthyophthirius multifiliis*, after an initial sublethal infection. Humoral antibodies in the fish can immobilize a number of surface membrane antigens of these parasites *in vitro* (Dickerson et al., 1989; Lin et al., 1996). There is interesting evidence of cross protection between a free-living ciliate and a parasitic flagellate in fish. Trout fry immunized with a flagellated protozoan, *Tetrahymena thermophila*, were protected from infections of *I. multifiliis* and *Ichthyobodo necator* (*Costia necatrix*) (Wolf and Markiw, 1982)**.** The presence of specific antibody in fish serum can be detected by using the gel immunodiffusion method (Harris, 1972) and the amount of antibody can be measured by using an enzyme-linked immunosorbent assay (ELISA) (Jones et al., 1993; Smith et al., 1992; Wongtavatchai et al., 1995).

1.5.2: Cell-Mediated Immunity

In addition to humoral antibody, cell-mediated immunity plays an important role against parasitic infestations (Cross and Matthews, 1993; Sharp et al., 1992). An aggregation of localized leucocytes is one criterion used to determine the response of cellular immunity (Cross and Matthews, 1993). In infections of pleurocercoids, neutrophils were the first leucocytes to engage the parasite, followed by the aggregation of macrophages which transformed into epitheloid cells (Sharp et al., 1992). The aggregation of leucocytes against pleurocercoids occurred during week 3-6 post-infection. Cross and Matthews (1993) found that immunized carp (*Cyprinus carpio*) challenged with *I. multifiliis* expressed a higher number of eosinophilic granular cells and basophils in infection sites than those in a primary infection. Greater localized phagocytosis by neutrophils, macrophages and resident

epidermal filament cells was also observed in immunized fish. They also found that pronephric leucocytes from immunized fish displayed enhanced non-specific phagocytosis.

Delayed type hypersensitivity is another criterion for determining the presence of cell-mediated immune response in fish (Thomas and Woo, 1990; Feng and Woo, 1996). T-cell-mediated delayed-type hypersensitivity (T_{DTH}) reaction has been detected in rainbow trout, *Oncorhynchus mykiss*, infected with *Cryptobia salmositica* (Thomas and Woo, 1990). The reaction at the infection sites consisted of an infiltration of mononuclear cells into the dermis and muscle layers, and the presence of edema. T_{DTH} has confirmed that infected, intact and thymectomized *O. mykiss* displayed significant in duration at the site of *C. salmositica* antigen injection at 8, 12 and 16 weeks post-infection (Feng and Woo, 1996). *In vitro* expression of cell-mediated immunity, an inhibition of macrophage migration, has been also detected in kidney cell suspensions incubated with *Cryptobia* antigen (Thomas and Woo, 1990). Partial protection against *C. salmositica* in naive rainbow trout has been shown after receiving leucocytes or plasma from immune fish (Jones and Woo, 1987). Thus, both cell-mediated immunity and humoral immunity play an important role against parasitic infection in fish.

Although immunity has been reported to be an important mechanism in preventing parasitic infections in fish, there are no reports of the immune response of fish to an infection of hexamitids. The present study provides information on immunity in angelfish infected with *Spironucleus vortens*.

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CHAPTER 2

IN VITRO STUDIES ON OPTIMAL REQUIREMENTS FOR THE GROWTH OF *SPIRONUCLEUS VORTENS*, AN INTESTINAL PARASITE OF THE FRESHWATER ANGELFISH[1]

2.1: ABSTRACT

Spironucleus vortens were cultivated in either an artificial medium at different temperatures or various pH conditions, or in medium supplemented with different bile concentrations at 25°C. Temperature, pH and bile requirements for the optimal growth of the parasite were determined. Parasites multiplied quickly at 28°C and 31°C and reached maximum numbers on day 4 of cultivation, whereafter they did not survive. At 25°C, parasites survived longer than those at 28°C and 31°C with no difference in multiplication rate during the exponential phase. The longest survival period was seen at 22°C, although the growth rate of the parasite was not as high as those at 25°C. At a higher temperature of 37°C, no parasites were observed alive after the second day of cultivation. Optimal pH range for the parasite's growth was 6.5 to 7.5, with the highest cell number at pH 7.5. Parasites survived longest (15 days) at pH 6.0, although the maximum number of cells was lower than those at the optimal pH. Parasites were dead within 24 h at pH levels above 8.5 or below 5.5. All cultures supplemented with either bovine or fish bile yielded numbers of parasites lower than cultures with no bile. In addition, parasite growth was significantly suppressed in medium supplemented with higher concentrations of bile. These results indicate that the optimal condition for the *in vitro* cultivation of *Spironucleus vortens* is 25°C and pH 6.5 to 7.5 without supplementation with bile.

[1] Accepted in Diseases of Aquatic Organisms 1999

2.2: INTRODUCTION

Spironucleus sp. are parasitic flagellates found in both freshwater and saltwater fish. They have been recorded in marine gadoids, Atlantic cod and haddock (Poynton and Morrison, 1990), and Atlantic salmon (Sterud et al., 1997). In freshwater fish, *Spironucleus sp.* have been recorded in grayling (Sterud et al., 1997), burbot (Sterud, 1998a), and cichlids, including angelfish (Kulda and Lom, 1964b; O'Brien et al., 1993; Poynton et al., 1995; Specht et al., 1989), and cyprinids (Molnár, 1974). The parasites have been located in the digestive tract or systemically in various organs of the fish. They can cause an enteritis, especially of the posterior intestine, varying in severity from a diffuse lymphoplasmacytic infiltration to a severe intestinal necrosis (O'Brien et al., 1993). *Spironucleus sp.* may reach the blood stream and liver by invasion across the injured intestinal wall, and are capable of causing severe parasitemia in a host under stress conditions (Molnár, 1974). It has been suggested that *Spironucleus spp.* cause systemic infection because they can invade intestinal mucosa and disseminate to other tissues (Siddall et al., 1992). In sea-caged Atlantic salmon *Salmo salar* L, *Spironucleus barkhanus* has been reported causing the death of fish, and the parasites were found in large numbers in most of the internal organs (Mo et al., 1990; Poppe et al., 1992).

Spironucleus vortens Poynton et al., 1995 was first successfully isolated from the freshwater angelfish (*Pterophyllum scalare*). The organism can be maintained and propagated in artificial medium (Poynton et al., 1995). A few studies have suggested the *in vitro* growth requirements of fish hexamitids (Poynton et al., 1995; Sterud, 1998b), but none have examined the specific optimal requirements for growth of *S. vortens*. Although *S. barkhanus* cultivated in bile-supplemented medium have shown a slight difference in

optimal temperatures between strains isolated from salmon and grayling (Sterud, 1998b), the optimal pH and bile requirement for *Spironucleus spp.* are still unknown. In addition, most of the *in vitro* cultivations of diplomonads, including fish hexamitids, have been done in culture medium supplemented with bovine bile (Buchmann and Uldal, 1996; Keister, 1983; Sterud, 1998b). To date, none of the fish hexamitids have been cultivated in medium supplemented with fish bile. In the present study, bile originating from bovine or fish were added to the culture medium and evaluated with temperature and pH for the optimal conditions maximizing the *in vitro* growth of *S. vortens*.

2.3: METHODS

2.3.1: Parasite Cultivation

Cryopreserved *Spironucleus vortens* were obtained from the American Type Culture Collection (ATCC No. 50386, Manassas, Virginia, USA). The parasites were thawed by immersion of the vial into water at 35°C for 2 minutes and the suspension (0.5 ml) immediately added to a 16 x 125 mm sterile screw-capped glass tube containing 13 ml culture medium. The culture tube was tightly closed and placed on a 15 degree horizontal slant in an incubator without light at 25°C. The parasites were propagated and subcultivated weekly by transferring 0.1 ml of parasite suspension to a sterile tube with 13 ml fresh culture medium.

2.3.2: Culture Medium

The flagellates were cultivated in sterile TYI-S-33 medium (ATCC No. 350-X) at pH 6.8 supplemented with the antibiotics, penicillin (2000 U ml^{-1}) and gentamicin (50 μg ml^{-1}).

2.3.3: Parasite Cell Counting

The concentration (average cell number ml^{-1}) of *S. vortens* in culture medium was determined daily by automatic cell counter (CASY®1; model TTC, Schärfe System GmbH, Germany). The parasite suspension was gently mixed and aliquots of 5 or 10 μl were added into counter containers with 10 ml PBS, pH 7.4.

2.3.4: Determination of Optimal Temperature

Culture medium (2 ml, pH 6.8) with *S. vortens* concentration of 5000 cells ml^{-1} were established in 4-ml screw-capped glass tubes. The parasites were incubated and allowed to grow in thermostat-regulated incubators without light at 22°, 25°, 28°, 31°, 34° and 37°C. The cell cultures at each temperature were performed in triplicate. Concentrations of *S. vortens* were determined every 24 h until no parasites were observed alive.

2.3.5: Determination of Optimal pH

Parasite cultures (30 ml, 5000 cells ml^{-1}) were established in 50-ml sterile tissue culture flasks. The pH of cultures was adjusted gradually from the original pH (6.8) to the appropriate final incubation pH: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 or 10.0

within 4 days of culture initiation. The pH of cultures were measured daily using a pH meter (Accumet® pH meter 10, Fisher Scientific, Pittsburgh, PA) and were adjusted to the final incubation pH by sterile 0.1 N NaOH or 0.1 N HCl. The culture flasks were tightly capped and incubated without light at 25°C. The average cell numbers of 3 subsamples were determined daily by the automatic cell counter as previously described.

2.3.6: Determination of Bile Requirement

Parasite cultures (2 ml, 5000 cells ml^{-1}) with different concentrations (0.00, 0.05, 0.20, 0.40, 0.80, 1.60, 3.20 and 6.40 mg ml^{-1}) of bovine bile (Sigma Chemical Co., No. B-8381) were established in 4-ml screw-capped glass tubes. Parasite cultures at each bile concentration were performed in triplicate and allowed to grow at 25°C. Concentrations of *S. vortens* were determined every 24 h until no parasites were observed alive. In addition to bovine bile, fish bile collected directly from the gall bladder of a species of cichlid (hybrid tilapia) was pooled and used as a supplement in the culture medium. Parasite cultures were established containing fish bile concentrations of 0 (control), 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.5%. Procedures for evaluating the parasite's fish bile requirement were done in the same manner as described for the bovine bile investigation.

2.3.7: Criteria for Determining Optimal Condition

Optimal conditions for the growth of the parasites were based on a combination of factors which included average cell number ml^{-1}, growth rate, survival time, and cell conditions (motility and morphology). The motility and shape of the parasites were observed under a compound microscope. Any condition of temperature, pH and bile that yielded high

numbers of active motile flagellates with a long survival period was considered an optimal condition.

2.3.8: Statistical Analysis

Growth rates of *S. vortens* during the exponential phase were analyzed and compared by a SAS statistical program (SAS Institute Inc., Cary, NC). Numbers of parasites during the exponential phase were transformed to ln-growth curve in order to stabilize the variances for analysis purpose. The exponential increases (slope) of growth and the number of parasites during the exponential phase were analyzed and compared by Tukey's HSD at $\alpha = 0.05$.

2.4: RESULTS

2.4.1: Optimal Temperature

Growth and average numbers of *S. vortens* cultivated under different temperatures are shown in Figure 2-1 and Table 2-1, respectively. No parasites were observed alive in the culture at 37°C after 24 h of culture initiation. At 28°, 31° and 34°C, parasites were observed alive for only the first 4 days of cultivation. The cultured cells, except those at 34°C and 37°C, started multiplaying with a lag phase followed by an exponential phase. The lag phases and the exponential phases of cultures at 25°, 28° and 31°C were one day shorter than those at 22°C. The cultures at 22°, 25°, 28° and 31°C yielded maximum cell numbers of 6.0, 6.0, 7.91 and 6.7 million cells ml^{-1}, respectively. The cultured cells at 25°C reached maximum cell numbers within 5 days, while those at 22°C reached the same cell number

within 8 days. However, cells in the cultures at 22°C were observed alive up to 10 days of the cultivation.

The ln-transformed growth curves of *S. vortens* during day 2 to day 4 of cultures are shown in Figure 2-2, and average numbers (backe-transformed mean) of the organisms during that period are shown in Table 2-2. An equation representing the growth of each curve is ln (cell no.) = $\beta_0 + \beta_1$day. The growth rate (β_1) at 22°, 25°, 28°, 31°, and 34°C during day 2 to day 4 were 1.52, 1.71, 1.84, 1.86, and 1.02, respectively. The parasites multiplied faster at 25°, 28° and 31°C than those at 22°C and 34°C. There was no significant difference between the proportional increase in cell numbers of cultures at 22°, 25°, 28°C and 31°C. Although the parasites multiplied at 22°C with no difference in growth rate than those at 25°, 28°, and 31°C, the number of parasites at 22°C during day 2 to day 4 was significant lower (Table 2-2). The parasites cultured at 22°C and 34°C also had similar cell numbers on the second and third day of cultivation, however, by day 4 the number of parasites at 22°C was significant higher than those at 34°C.

Under microscopic observation, *S. vortens* cultivated at temperature ranges from 22°C to 31°C were not different in shape. The majority of the cells were pyriform in shape with a low percentage of multinucleated trophozoites. The dividing trophozoites which resembled bifurcated poles (V-shape cells) were found in the highest number on the second day of cultivation. In addition, the dividing trophozoites which were round to oval shape were also observed in the culture medium. The dividing trophozoites did not always move forward in one direction, but sometimes in an opposite direction. A high percentage (12-17%) of abnormal trophozoites was seen in the culture at 34°C. The parasites at each temperature

actively moved forward in the culture medium, and were less active only the last few days of cultivation.

2.4.2: Optimal pH

Growth and average numbers of *S. vortens* cultivated under different pH conditions is shown in Figure 2-3 and Table 2-3, respectively. Parasites had no difference in growth rates during the first 2 days of cultivation. The fastest growth rates were observed between pH of 6.5 and 7.5. The parasites cultivated at pH 6.5 and 7.0 were similar in growth patterns during the first 6 days of cultivation, and they had one day shorter period of multiplication compared to those incubated at pH 7.5. However, the highest average cell number (12.08 x 10^6 cells ml^{-1}) was observed in culture at pH 7.5. The lowest pH that the organisms had a moderate growth rate and a long survival period was 6.0. Parasites survived only a few days at pH 5.5, 8.0 and 8.5 and were killed within 24 h at a pH above 8.5 or below 5.5.

2.4.3: Bile Requirements

Spironucleus vortens in medium (pH 6.8) supplemented with bovine bile concentrations lower than 3.2 mg ml^{-1} multiplied slowly during the first 3 days of cultivation. Parasites multiplied gradually with the highest growth rate during day 4 to day 5. The cultured cells in the medium supplemented with bile concentrations lower than 1.6 mg ml^{-1} had similar replication rates after day 5 of cultivation. All cultures supplemented with bovine bile had maximum numbers of cells lower than the control culture with no bile (Fig. 2-4, Table 2-4). Cell growth was suppressed at high concentrations (3.2 and 6.4 mg ml^{-1}) of bovine bile. The numbers of cells at these higher concentrations of bile gradually decreased from the first day of cultivation until no cells were observed alive after day 5.

The parasites were also suppressed in medium supplemented with fish bile (Fig. 2-5). The average numbers (Table 2-5) of parasites ml^{-1} at all concentrations of fish bile were lower than those in the control culture with no bile. Parasites cultivated in fish bile-supplemented (1-8%) medium multiplied slowly during the lag phase, and no cells were observed alive after they reached peak numbers. In cultures supplemented with 10% fish bile, the highest number of parasites was 6.3×10^3 cells ml^{-1}, and only 200 cells ml^{-1} of parasites were observed alive on the last day of cultivation. At 12.5% fish bile supplementation, the number of parasites in cultures gradually decreased and no cells were observed alive on day 4 of cultivation.

2.5: DISCUSSION

Spironucleus vortens are highly motile flagellates. They actively move forward in the culture medium at temperatures ranging from 22°C to 34°C. Although *S. vortens* has been reported to grow and reproduce between 25°C and 31°C in culture medium (Poynton et al., 1995), the present study broadens that temperatures range to a low of 22°C and a high of 34°C. At 22°C the parasites are less active via microscopic observation than those at the higher temperatures, but survive longer. This suggests that the long-term risk of maintaining a *Spironucleus* infection in angelfish may be increased when the environmental temperature is decreased to 22°C.

In the artificial medium, normal pyriform-shaped trophozoites were the dominant form (Fig. 2-6) seen throughout the cultivation period at all temperatures; and they have been reported to be the typical form of the free flagellate found in the intestinal lumen of angelfish (Poynton et al., 1995). The dividing trophozoites (Fig. 2-7, Fig. 2-8) were found in the

highest number on the second day of cultivation. Irregular (abnormal) multinucleated trophozoites (Fig. 2-9) were also found in low numbers throughout the cultivation period, with a higher percentage (12-17%) in the culture at 34°C suggesting that high temperature (34°C) is a factor inducing failure of cytokinesis of the parasite resulting in a decrease in the number of new free-flagellated trophozoites. Cysts were not observed in any of the cultures. To date, cysts of fish hexamitids are very rare either in cell culture or in host feces. They were reported in the study of *Hexamita salmonis* in trout (Moore, 1922; Sano, 1970), and during *in vitro* cultivation (Uldal, 1996), but were not detected in other recent studies (Kent et al., 1992; Kulda and Lom, 1964a; Tojo and Santamarina, 1998). Therefore, it is probable that free flagellate trophozoites, not cysts, of fish hexamitids play an important role in disease transmission in a natural condition.

The optimal temperature for *S. vortens* growth in this study was determined to be 25°C because the parasites had a high replication rate, good motility, and extended survival period of up to 6 days. Although at 22°C the organisms survived longest and had no difference in growth rate compared to those at 25°C, they were less active throughout the culture period and required longer period to reach a maximum number. At 28°C and 31°C, the parasites had the high replication rate, reached maximum numbers by day 4, but did not survive after that time. Either a shortage of nutrients and/or increased waste products released from live or dead cells are factors that may have contributed to the death of the parasites. In addition, temperature was considered to be the primary factor in the death of *S. vortens* in cultures maintained at 34°C or higher. The parasite's growth was significantly suppressed and was lethal within 24 h at 37°C.

Fish are poikilothermic animals which adapt themselves correspondingly to changes in water temperature. Freshwater angelfish (*Pterophyllum scalare*) are indigenous in a tropical zone, South America: Amazon River (Axelrod, 1985), and have been successfully bred and maintained in captivity under warmwater conditions. The most suitable temperature for raising angelfish ranges from 22°C to 30°C (Mills et al., 1988). Therefore, the present study indicates that an appropriated temperature for raising angelfish is also suitable for facilitating the growth of *S. vortens*. It has been suggested that high temperatures (above 28°C) can sometimes help in controlling hexamitid infections (Bassleer, 1983). The present study supports this suggestion in that the trophozoites of *S. vortens* are suppressed and rapidly killed at high temperature. Thus, maintaining higher water temperatures may help decrease the number of trophozoites in the host and might minimize the rate of infection.

There were few studies reporting the optimal conditions for fish hexamitids. The optimal temperature and pH for *Hexamita salmonis* isolated from rainbow trout were 10°C and pH 7.5 to 8.0 (Buchmann and Uldal, 1996). *Spironucleus barkhanus* isolated from grayling and salmon have a suitable temperature range for growth from 5°C to 20°C (Sterud, 1998b). Both of these organisms are parasites of coldwater fish. In the present study, the suitable temperature range for *S. vortens* is 22°C to 25°C which is higher than the previously mentioned species. However, optimal pH (6.5-7.5) for the growth of *S. vortens* is closed to that reported for *H. salmonis*. Thus, it is obvious that different species of hexamitids have different optimal conditions for growth due to the adaptation of the hosts to environmental conditions.

Bile concentrations between 0.03-0.96 mg ml^{-1} have been reported to stimulate growth of *Hexamita salmonis* cultivated in artificial medium (Buchmann and Uldal, 1996). However, the present study demonstrated that bile was not a requirement for the growth of *S. vortens*. In addition, the growth of the parasite was significantly suppressed at higher concentrations of either bovine (3.2 and 6.4 mg ml^{-1}) or fish (10% and 12.5%) bile. *Spironucleus sp.* have been reported frequently to cause diseases in many aquarium fishes (Molnár, 1974) including the freshwater angelfish (O'Brien et al., 1993). The parasites are normally found in fish intestine (O'Brien et al., 1993; Poynton et al., 1995), but may be found in other organs including the gall bladder (Molnár, 1974; Sterud, 1998b). However, the results of this study indicate that *S. vortens* are less tolerant to bile, therefore, suggesting that they may only accidentally enter the gall bladder during heavy intestinal or systemic infections.The *in vitro* environmental conditions such as temperature, pH and concentration of bile are all important factors in maximizing or suppressing the growth of the parasite. In this study, a temperature of 25°C and pH between 6.5 to 7.5 were considered as the optimal conditions for the parasite growth. However, *Spironucleus vortens* cultivated in culture medium at 25°C during routine cultivation (13 ml) and in the pH experiment (30 ml) survived longer than those cultivated in the temperature experiment (2 ml). Thus, the amount of nutrient (medium) provided to the organisms may also be considered an important factor in the growth and survival requirements.

Spironucleus vortens has been found mostly in the middle to posterior region of angelfish's intestine (Poynton et al., 1995). The present study is useful in explaining the microhabitat of *S. vortens*. The anterior intestine, which presumably contains a higher concentration of bile, is not suitable for long-term parasite survival and the number of parasites should be lower in this region. Conversely, the number of parasites should be

higher in the middle and posterior intestine due to the lower concentration of bile in these areas.

2.6: ACKNOWLEDGMENTS

I wish to express thanks to Dr. Sarah L. Poynton for helpful suggestions on this research, and to Daniel Ward for help with the statistical analysis.

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CHAPTER 3

EFFICACY OF VARIOUS CHEMOTHERAPEUTIC AGENTS ON THE GROWTH OF *SPIRONUCLEUS VORTENS*, AN INTESTINAL PARASITE OF THE FRESHWATER ANGELFISH [1]

3.1: ABSTRACT

Seven chemotherapeutic agents (dimetridazole, metronidazole, albendazole, fenbendazole, mebendazole, pyrimethamine, and magnesium sulfate) were examined for growth inhibition on the cultivation of *Spironucleus vortens*. Dimetridazole and metronidazole were effective in inhibiting the parasite's growth. At concentrations of 1 µg ml^{-1} or higher, both dramatically decreased numbers of parasites. At 24 h exposure, 33% of parasites were inhibited when exposed to dimetridazole or metronidazole at concentrations of 2 and 4 µg ml^{-1}, respectively. Dimetridazole at 4 µg ml^{-1} or higher concentrations decreased the number of organisms to 50% or less after 48 h exposure. During the same period of time, the numbers of parasites decreased to 50% or less when exposed to metronidazole at 6 µg ml^{-1} or higher. Pyrimethamine at concentrations of 1 to 10 µg ml^{-1} was not effective in inhibiting the parasite's growth. Albendazole and fenbendazole at concentrations of 0.1 and 0.5 µg ml^{-1} were similar in inhibiting the growth of the organism. Both compounds suppressed parasite growth at concentrations of 1.0 µg ml^{-1} or higher after 24 h exposure. Mebendazole inhibited the parasite's growth at concentrations of 0.5 µg ml^{-1} or higher. At 72 h exposure, 45-50% of the parasites were inhibited when exposed to mebendazole at concentrations higher than 0.5 µg ml^{-1}. Magnesium sulfate at concentrations of 70 mg ml^{-1} or higher also suppressed the growth of parasites after 24 h exposure. These results indicate that

[1] Published in Diseases of Aquatic Organisms 1999, 38(1): 47-52.

dimetridazole, metronidazole and mebendazole are the most effective chemotherapeutic agents *in vitro* at inhibiting the growth of *S. vortens*.

3.2: INTRODUCTION

Hexamita and *Spironucleus* are diplomonad flagellates frequently found in the digestive tract of both freshwater and saltwater fish (Ferguson, 1979; Kulda and Lom, 1964; Mo et al., 1990). These motile flagellates can cause cellular damage in the intestinal tract of infected fishes and heavy infections may interfere with normal growth of the host (Yasutake et al., 1961). They often cause disease when the host has a low resistance or is adversely affected by some predisposing factors such as inadequate nutrition, low oxygen content, poor sanitation or overcrowding (Lom and Dyková, 1992). They have been reported as pathogens causing enteritis and mortalities in salmonids, cyprinids and ornamental tropical aquarium fish (Gratzek, 1988; Sommerville, 1981; Woo and Poynton, 1995). Thus, treatment and control of these organisms are important in both the foodfish and tropical fish industry. Most studies of chemotherapeutic agents for this group of parasites have been evaluated using *Hexamita sp*., a parasite of salmonids (McElwain, 1968; Tojo and Santamarina, 1998; Yasutake et al., 1961). Although there is a report of chemotherapeutic activity on *Spironucleus muris*, a parasite of the mouse intestine (Oxberry et al., 1994), there are no published reports of chemotherapeutic efficacy on *S. vortens* of fish.

Metronidazole, 1-(2-hydroxy ethyl)-2-methyl-5-nitroimidazole, is the active compound of nitroimidazole used in the treatment of infections caused by anaerobic bacteria and protozoa (Amon et al., 1978; Baines, 1978; McLean et al., 1984). The drug is rapidly absorbed from the gastrointestinal tract and effective against obligate anaerobic organisms having an active

hydrogenase (Baines, 1978; Church et al., 1996). It has been applied frequently in the treatment of flagellate infections causing urogenital trichomoniasis and giardiasis in human (Catchpool, 1980). In addition, metronidazole (Flagyl®) and metronidazole related compounds (Ipropran® and Emtryl®) have been used in the treatment of hexamitiasis in trout, ornamental fish and several species of cichlids (Gratzek, 1983). Therefore, metronidazole and dimetridazole, a metronidazole-related compound, may be effective in the treatment of *Spironucleus vortens*, an aerotolerance anaerobe in angelfish.

The benzimidazole anthelmintic is another group of chemotherapeutics that has been shown to be effective against intestinal protozoa such as *Giardia lamblia* (Bell et al., 1993; Edlind et al., 1990), *G. duodenalis, Trichomonas vaginalis* and *Spironucleus muris* (Oxberry et al., 1994). Being less absorbable, albendazole and mebendazole were 30 to 50 times more active than metronidazole (Edlind et al., 1990). Albendazole has also been reported to be successful in the treatment of *Hexamita salmonis* infection in rainbow trout (Tojo and Santamarina, 1998). The target of benzimidazoles in *Giardia sp.* is a structure called β-tubulin (Edlind et al., 1990). The internal structures of *S. vortens* such as recurrent flagella, peripheral ridges and body (beneath the cell surface) are also supported by microtubules (Poynton et al., 1995). Therefore, benzimidazoles may be effective in the treatment of *S. vortens*.

Pyrimethamine (2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine) is an antiprotozoal drug commonly used for antimalarial activity and the treatment of toxoplasmosis (Bradyley and Marciano-Cabral, 1995; Pratt and Fekety, 1986; Rosenblatt, 1992). Pyrimethamine in combination with sulfadiazine has also been used in the successful treatment of chronic intestinal coccidiosis in man (Trier et al., 1974). The drug is chemically related to

trimethoprim and is slowly absorbed from the gastrointestinal tract (Catchpool, 1980). Parasiticidal efficacy of pyrimethamine is due to inhibition of the synthesis of dihydrofolate reductase which catalyzes the conversion of dihydrofolate into tetrahydrofolate in organisms (Bradyley and Marciano-Cabral, 1995; Pratt and Fekety, 1986). The folate coenzyme, tetrahydrofolate, is necessary in the reactions involved in purine, pyrimidine and amino acid synthesis (Bradyley and Marciano-Cabral, 1995; Pratt and Fekety, 1986). Thus, organisms exposed to pyrimethamine are killed due to a deficiency of the folate coenzyme that results in the inhibition of cell division (Catchpool, 1980). With the energetic self-division of *S. vortens*, pyrimethamine may be effective in the treatment of spironucleosis.

Unlike the previously discussed chemotherapeutic agents, magnesium sulfate ($MgSO_4$, Epsom salt) is frequently used as purgative. It has been used for the treatment of parasitic diseases, especially luminal parasite infestations. When the magnesium salts reach the intestine, magnesium ions cause the release of cholecystokinin, which increases peristaltic activity of the intestine (Jenkins, 1988). Increased movement then helps the host to expel or decrease the number of parasites in the intestine. Free flagellates of diplomonads are the most commonly encountered stage found in the fish's intestine (Woo and Poynton, 1995) and are capable of disseminating to other tissues during stressful conditions of the host (Molnár, 1974). Therefore, it was hypothesized that magnesium sulfate may also be helpful in reducing the number of parasites in the intestine of the fish.

In the present study, seven different chemotherapeutic compounds were examined at various concentrations for any effect on the ability of the parasite, *S. vortens,* to survive and reproduce in an *in vitro* culture system.

3.3: METHODS

3.3.1: Parasite Cultivation

Spironucleus vortens were cultivated in TYI-S-33 medium (ATCC, Cat. No. 350-x) at pH 6.8. The antibiotics, penicillin (2000 U ml^{-1}) and gentamicin (50 µg ml^{-1}), were added to the medium during routine culture, but were omitted during experimental treatments. The organisms were cultivated in a sterile flask and placed in an incubator at 25°C without light.

3.3.2: Parasite Cell Counting

The concentration (average cell number ml^{-1}) of *S. vortens* in the culture medium was determined daily by automatic cell counter (CASY®1; model TTC, Schärfe System, GmbH, Germany). The parasite suspension was gently mixed, aliquots of either 5 µl or 10 µl were added into counter containers with 10 ml PBS, and the resulting cell mixture analyzed.

3.3.3: Growth Inhibition Assay

All chemicals, except magnesium sulfate, were prepared at different concentrations by dissolving in dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO). Concentrations of Epsom salt ($MgSO_4$) were prepared by dissolving in fresh culture medium. Chemotherapeutic agents were divided into three separated testing groups. Chemicals in the first group were dimetridazole, metronidazole and pyrimethamine; the second group were albendazole, fenbendazole and mebendazole; and the last group was

magnesium sulfate. Control groups were established for each chemotherapeutic testing group, and an additional group was established to determine the effect of DMSO (solvent) on the parasite's growth. All cultures were established in triplicate in sterile 4-ml screw capped glass tubes which were placed in a thermo-regulated incubator at 25°C. The average number of parasites (cells ml^{-1}) was determined every 24 h until no parasites were observed alive.

Dimetridazole, metronidazole and pyrimethamine were tested at concentrations of 1, 2, 4, 6, 8 and 10 μg ml^{-1}. Log-phase *S. vortens* concentration of 5.44 x 10^6 cells ml^{-1} was established on the first day of cultivation with 2 ml of culture medium per vial and incubated at 25°C without light. Albendazole, fenbendazole and mebendazole were examined at concentrations of 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 μg ml^{-1}. Log-phase *S. vortens* concentration of 4.23 x 10^6 cells ml^{-1} was established on the first day of cultivation with 2 ml of culture medium per vial and incubated as described. Magnesium sulfate was prepared at concentrations of 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg ml^{-1}. Log-phase parasites were established in 2 ml of culture medium per vial at 3.78 x 10^6 cells ml^{-1} on the first day of cultivation. The cultures were incubated as described. DMSO (solvent) was examined for its effect on the parasite's growth. Cultures with different concentrations of DMSO (0.025, 0.05, 0.10, 0.15, 0.20 and 0.25%) were established and examined along with the experiment of dimetridazole, metronidazole and pyrimethamine.

3.4: RESULTS

Growth and average cell numbers of *S. vortens* at different concentrations of DMSO are shown in Figure 3-1 and Table 3-1, respectively. DMSO dissolved in the artificial medium at concentrations of 0.025% to 0.25% minimally affected the growth of *S. vortens*. Compared to the growth of organisms in cultures with DMSO, pyrimethamine at concentrations of 1 to 10 μg ml^{-1} also did not inhibit the growth of the parasites (Fig. 3-2, Table 3-2). Under microscopic observation, the conditions (morphology and movement) of organisms in cultures with DMSO or pyrimethamine were not different from those of the control (medium only) cultures.

Dimetridazole weakly inhibited the organism's growth at a low concentration (1 μg ml^{-1}) (Fig. 3-3, Table 3-3). However after 24 h incubation with 2 μg ml^{-1} dimetridazole, 33.46% of the parasites displayed inhibited growth. The percentages of parasites which were inhibited to grow were not different from those exposed to dimetridazole at 4 μg ml^{-1} or higher concentrations. More than 50% of the organisms were killed after 48 h exposure to dimetridazole at concentration 4 μg ml^{-1} or higher. Under microscopic observation, a large number of trophozoites of *S. vortens* exposed to dimetridazole were not in their normal pyriform shape, but were multinucleated trophozoites that were larger in size with numerous flagella.

Growth inhibition of *S. vortens* by metronidazole was similar to that observed with dimetridazole (Fig. 3-4). At 24 h exposure, 33.09% of parasites were dead after incubation with 4 μg ml^{-1}. Metronidazole at concentrations higher than 4 μg ml^{-1} decreased the number of parasites (Table 3-4) by more than 50% at 48 h exposure. Compared to dimetridazole,

metronidazole was less effective in decreasing the number of parasites at the same concentration and exposure period. Metronidazole (2 μg ml^{-1}) killed only 17.46% of the parasites, whereas 33.46% of the organisms were killed with dimetridazole at the same concentration and same exposure period (24 h).

Growth inhibitions of *S. vortens* by albendazole and fenbendazole are shown in Figure 3-5 and Figure 3-6, respectively. Albendazole and fenbendazole failed to inhibit the parasite's growth at concentrations less than 0.5 μg ml^{-1} (Table 3-5, Table 3-6). At higher concentrations (1-3 μg ml^{-1}), both albendazole and fenbendazole minimally inhibited the growth of the organism. Compared to albendazole and fenbendazole, mebendazole (Fig. 3-7) was more effective in inhibiting the parasite's growth. Mebendazole inhibited the parasite's growth at concentrations of 0.5 μg ml^{-1} or higher. The numbers of parasites (Table 3-7) gradually decreased throughout the cultivation period when exposed to an increasing concentration of mebendazole, with the greatest period of growth inhibition seen after 24 h exposure. After 72 h of exposure, approximately 45-50% of the parasites were killed when incubated with mebendazole (1.0 to 3.0 μg ml^{-1}).

Growth and average cell numbers of *S. vortens* in different concentrations of magnesium sulfate are shown in Figure 3-8 and Table 3-8, respectively. Number of parasites in the control culture was higher than those in any cultures with $MgSo_4$. Epsom salt at all concentrations (5-100 mg ml^{-1}) affected the growth of *S. vortens*. The average numbers of parasites at concentrations of 5 to 60 mg ml^{-1} gradually increased with exposure time, but gradually decreased at 70 mg ml^{-1} or higher of $MgSO_4$ after 24 h exposure.

3.5: DISCUSSION

Spironucleus vortens, aerotolerance anaerobes, are highly motile flagellate protozoa. They are normally pyriform in shape and they actively move in a forward direction in the culture medium. In the present studies *S. vortens* were cultivated and examined in an artificial medium with different types and concentrations of chemotherapeutic agents. Nitroimidazole, particularly metronidazole, is the most widely prescribed drug in the treatment of anaerobic protozoa (Amon et al., 1978; Church et al., 1996; Edwards, 1980). Compounds in this group enter cells and are accumulated inside the cells by passive diffusion without an active transport process (Chapman et al., 1985; Müller, 1983). Their intermediate products, the reduced nitro-group forms, function as a short-lived cytotoxic effect, and the accumulation of these intermediates enhances the rate of entry of the chemical into the susceptible cells (Edwards, 1980). Metronidazole also reduced the homogeneous material within the cytoplasm and caused misshapen trophozoites of *S. muris*; but did not affect the cytoskeleton (Oxberry et al., 1994). Metronidazole at 5 ppm in water (Bassleer, 1983; Gratzek, 1983; Jenkins, 1988) or at 10 mg in 1.0 g of food (Stoskopf, 1988) is recommended for the treatment of intestinal flagellates in fish.

The present studies showed that trophozoites of *S. vortens* were inhibited and misshapen in the cultures exposed to dimetridazole or metronidazole. This may have been caused by the toxicity of the intermediates accumulated in the cells and the reducing of cytoplasmic materials. It has been proposed that the toxicity of the intermediates is due to their interaction with DNA, decreasing the stability and integrity of DNA, and possibly with other macromolecules (Edwards, 1980; Müller, 1983). These radical anions generated within intact hydrogenosomes also may damage the radical-generating system and the

hydrogenosome membrane, and are capable of efflux from isolated organelles (Chapman et al., 1985). The results of misshapen or damage cell organelles may also lead to a failure of cell division. Thus, the large number of irregular, multinucleated trophozoites found in cultures exposed to dimetridazole and metronidazole may have been a result of cell division failure. Nitroimidazoles (metronidazole, benznidazole, ronidazole and secnidazole) have been reported to be an effective chemotherapeutants eliminating *H. salmonis* in infected rainbow trout (*Oncorhynchus mykiss*) (Tojo and Santamarina, 1998). The present study also showed that the nitroimidazoles (dimetridazole and metronidazole) were effective in growth inhibition of *S. vortens*, however, the determination of specific dosages and their efficacy *in vivo* for *Spironucleus sp.* in various fishes are still needed.

Trophozoites of *S. vortens* cultivated in medium with the benzimidazoles were also misshapen. However, the number of malformed trophozoites and multinucleated trophozoites were lower than those incubated with the nitroimidazoles. At the same concentration, mebendazole was more effective than albendazole and fenbendazole in inhibiting the growth of *S. vortens*. The effect of benzimidazoles on parasites is different from nitroimidazole in that the target of benzimidazole is a structure called β-tubulin (Edlind et al., 1990). Mebendazole has been shown to damage β-tubulin by distorting cell structures and rapidly disrupting the ventral disk and flagella of *G. lamblia* (Edlind et al., 1990), while albendazole binding to the cytoskeletal proteins causes trophozoites of *G. duodenalis* to become swollen, misshapen with cytoplasmic dark granule materials and finally die (Oxberry et al., 1994). Albendazole also increased glycogen granules and cytoplasmic vesicles and caused thickening of the parasite's cell coat with many invaginations (Oxberry et al., 1994). However, albendazole had no effect on the cytoskeleton or any other gross morphological characteristics of *S. muris* (Oxberry et al., 1994).

Pyrimethamine is most widely used clinically as an antimalarial chemotherapeutic agent (Pratt and Fekety, 1986). The drug has a parasiticidal effect by inhibiting the process of cell division. In the present study, pyrimethamine was examined for its efficacy on the growth of an energetic flagellated diplomonad, *S. vortens*. The results indicated that pyrimethamine did not affect the cell division of trophozoites. It is suggested that the parasites may not need the coenzyme, tetrahydrofolate, in the cell division process or the parasites may have alternative pathways that help in purine, pyrimidine and amino acid synthesis. Magnesium sulfate (Epsom salt) minimally affected the parasite's growth at low concentrations but inhibited growth at concentrations higher than 60 mg ml^{-1}. It is suggested that magnesium sulfate at high concentration may interfere with homeostasis of ions between the inside and the outside of the cell. Further information of the mechanism actions of pyrimethamine and magnesium sulfate on diplomonads is needed.

Of the seven chemotherapeutic agents investigated in this study, dimetridazole, metronidazole and mebendazole were the most effective compounds *in vitro* at inhibiting the growth of *S. vortens*. Results of this study are useful for the treatment of spironucleosis in angelfish, and may be useful for the treatment of diplomonads in other species of fishes. However, the success of individual treatments was dependent on both drug concentration and time of exposure.

3.6: ACKNOWLEDGMENTS

I wish to express thanks to Dr. David S. Lindsay for providing advice and some of the chemicals used in this research, and to Dr. Sarah L. Poynton for invaluable encouragement and insight into this research.

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CHAPTER 4

PATHOGENESIS OF *SPIRONUCLEUS VORTENS* INFECTION IN ANGELFISH

4.1: ABSTRACT

Pathogenesis of spironucleosis was studied in angelfish orally (PO) infected and intraperitoneally (IP) inoculated with *Spironucleus vortens*. One hundred and twenty adult angelfish were divided into two sets of control fish and three sets of experimental fish. The experimental fish were orally infected with 5×10^6 or 1×10^7 cells of *S. vortens*, or intraperitoneally injected with 2×10^6 cells of the organisms. The control angelfish were gavaged or IP injected with PBS on the same day. The control angelfish were in normal body condition throughout the experimental period. There were no pathological changes observed in the intestines or internal organs of the control angelfish at any time during the study. The orally infected angelfish were inappetent with no other clinical signs. Systemic spironucleosis was observed in angelfish IP inoculated with the organisms. Twenty percent of the IP-infected angelfish died during the first three weeks of infection after they showed clinical signs of inappetite, weakness, respiratory distress and laying on their sides. There were no external lesions observed in any angelfish. *Spironucleus vortens* were found only in the intestinal lumen of the PO-infected angelfish, however, they were observed in blood circulation, gills, peritoneal cavity, spleen, ovary, gall bladder, stomach, and intestine of the IP-infected angelfish. Examination of the angelfish's intestines revealed mild to moderated multifocal enteritis with infiltration of eosinophilic granulocytes and lymphocytes in the submucosa and mucosa, respectively. No parasites were observed in the intestinal mucosa although they were found in gastric submucosa of the IP-infected angelfish. Within the

abdominal cavity, the organisms caused peritonitis and invaded the abdominal peritoneal lining, and occasionally in the mesenteric blood vessels. Histologic examination revealed mild to marked granulomatous inflammation in a wide variety of the internal organs of IP-infected angelfish. Macrophages, lymphocytes and plasma cells were the inflammatory cells found most commonly in the internal organs. In the ovary, *S. vortens* were observed in the interfollicular interstitium, inside follicles, and in blood vessels. Histopathologic lesions in the kidney revealed nephrocalcinosis and glomerulosclerosis accompanied by reactive granuloma formation. Mild inflammation and decreasing hepatocyte vacuolization were observed in the hepatopancreas. In the spleen, pathological examination revealed telangiectasis, and mild fibrosis at the perimeter of melano-macrophage centers. It is suggested that *S. vortens* normally inhabits in the intestine where it causes mild enteritis. However, if *S. vortens* reaches the abdominal cavity and disseminates to other organs via blood circulation, then the organism may cause systemic granulomatous inflammation and eventually the death of the host.

4.2: INTRODUCTION

Hexamitids are parasitic protozoa classified in the Order Diplomonadida, Phylum Sarcomastigophora. They are extracellular flagellates with direct life cycles and are normally found in the intestinal tract of vertebrates (Woo and Poynton, 1995). In fish, hexamitids are the most commonly encountered parasites that cause disease of both local and systemic infections with significant morbidity and mortality (Kent et al., 1992; Molnár, 1974; Poppe et al., 1992). Trophozoites, free flagellated parasites, are the major stage found in the intestinal lumen (Woo and Poynton, 1995). They are found in a preferred location in the intestine where it is assumed to have suitable environmental conditions for them. However,

the parasites can be found throughout the entire length of the intestinal tract in a severe infection (Uldal and Buchmann, 1996), and they may disseminate to various organs including liver, muscle and gall bladder in a systemic infection (Molnár, 1974). In sea-caged Atlantic salmon *Salmo salar* L, *Spironucleus barkhanus* has been reported causing death of the fish where the parasites were found in large numbers in most internal organs (Mo et al., 1990; Poppe et al., 1992). *Spironucleus spp.* have been reported in both freshwater (O'Brien et al., 1993; Poynton et al., 1995; Sterud, 1998; Sterud et al., 1997) and saltwater fish (Poynton and Morrison, 1990; Sterud et al., 1998). Although the organisms generally inhabit the fish intestine as commensal protozoan, they can become pathogenic and provoke disease in lowered resistance hosts (Lom and Dyková, 1992). In common carp, an incidence of spironucleosis is rare while in angelfish (Specht et al., 1989) and some other ornamental fishes the disease occurs frequently (Lom and Dyková, 1992). In infected angelfish, *Spironucleus spp.* caused enteritis with vary severity from a diffuse lymphoplasmacytic infiltration of the lamina propria to a severe necrotizing reaction of the intestinal tract (O'Brien et al., 1993). Systemic spironucleosis caused losses in angelfish stock up to 50% (Molnár, 1974), and it is believed that only *Spironucleus spp.* cause systemic infection because they can invade intestinal mucosa and disseminate to other tissues (Siddall et al., 1992). Although *Spironucleus spp.* causes disease in several economically important fish species and in many regions of the world, their pathogenicity is not well understood (Woo and Poynton, 1995). In the present study, the pathology caused by *Spironucleus vortens* was examined in experimentally infected freshwater angelfish and the role of this organism causing pathogenicity in fish was discussed.

4.3: METHODS

4.3.1: Fish (angelfish, *Pterophyllum scalare*)

Adult angelfish (Fig. 4-1) (120 fish) with an average weight of 8.74 ± 2.19 g were kept at 28°C in 20 gallon tanks (15 fish each) with a biofilter system and aerated circulation. The angelfish were previously treated with metronidazole (50 ppm) twice at 2 weeks interval to eliminate the possibility of any diplomonads in the intestines, and no flagellates were observed at any time after the treatment. The angelfish were fed twice a day with a commercial flake diet (TetraMIN®, Tetra Sales, Blacksburg, VA) and they were allowed to acclimate at least 2 weeks before starting the experiment. The angelfish were separated into 2 groups; control group (30 fish) and experimental group (90 fish). All fish were kept in the same room with the same environmental conditions (temp, lighting, etc.).

4.3.2: Infection of *Spironucleus vortens*

Spironucleus vortens grown in TYI-S-33 medium (ATCC) were harvested during the log phase from the routine culture. They were prepared by centrifugation at 360 x g for 7 min, washing 3 times with PBS (pH 7.4), and counted by using an automatic cell counter (CASY®1; model TTC, Schärfe System GmbH, Germany). The angelfish were anaesthetized with tricane methanesulfonate (MS-222, Sigma Chemical Co., St. Louis, MO) (Fig. 4-2, 4-3). Infection of the *S. vortens* was performed by oral administration (PO) or intraperitoneal (IP) injection on the first day of experiment (d1). Using a micropipette tip attached with a small plastic tube (Fig. 4-4, 4-5), angelfish of two experimental groups (30 fish each) were orally inoculated with 5×10^6 (PO1) and 1×10^7 (PO2) organisms of

S. vortens (Fig. 4-6). Another 30 angelfish of a third experimental group were intraperitoneally injected with 2 x 10^6 organisms of *S. vortens* (IP) (Fig. 4-7). The control fish were gavaged (15 fish) and intraperitoneally injected (15 fish) with PBS (pH 7.4) on the same day. After inoculation and gavaging, each group of fish were immediately returned to separate tanks of water and observed for any abnormal behavior or clinical signs induced by the infection.

4.3.3: Clinical Observation

Fish were observed daily for abnormal clinical signs of inappetite, emaciation, anemia, lethargy, abnormal coloration, abdominal distension, exophthalmia, pseudocast feces and abnormal swimming patterns. Morbidity and mortality of fish were recorded and compared between the control and the experimental groups.

4.3.4: Gross Pathological Examination

Six angelfish (3 orally dosed and 3 IP dosed) of the control group and 6 angelfish of each experimental group were sacrificed on d7, d14, d21, and d28. The fish were euthanized using an overdose of MS-222 and killed by cervical separation. Fish were cut along the abdominal lining to expose all internal organs. Any pathological changes (size, color, shape, secretion, and consistency) were observed and recorded. The fish's intestines, internal organs (liver, spleen, kidney) and reproductive organs (ovary and testis) were collected and processed for either the light microscopic or electron microscopic examinations as described in the next sections.

4.3.5: Light Microscopic Examination

Intestines from 2 fish of the control group and 3 fish of each experimental group were collected on d7, d14, d21 and d28. The entire gastrointestinal tract was removed, measured for length, cut into 3 equal portions, and fixed in 10% neutral buffered formalin. Prior to fixing, some pieces of the intestines were removed, squashed on glass slides, and observed for the parasites using a light microscope. Internal organs (spleen, kidney, liver, and reproductive organs) of the angelfish were also fixed in 10% neutral buffered formalin. The fixed tissue samples were embedded in paraffin and cut into 6 μm thin sections. The tissue sections were stained with either hematoxylin & eosin (H&E) or a Feulgen stain. Pathological changes of angelfish tissues were observed under a light microscope. In addition to the histopathological examination, fresh smears of angelfish blood and bile were observed under a light microscope for the presence of parasites.

4.3.6: TEM and SEM Examination

Intestines from 1 fish of the control group and 3 fish of each experimental group collected on d7, d14, d21, and d28 were processed for scanning electron microscope (SEM) and transmission electron microscope (TEM) ultrastructural studies. The intestine was removed from the fish and measured for the length. Cold (4°C) fixative (5% glutaraldehyde, 4.4% formaldehyde, 2.75% picric acid in 0.05 M sodium cacodylate buffer at pH 7.4) was injected into the lumen of anterior, middle, and posterior portions of the intestine. The intestine was then cut into 3 equal portions and placed in the cold fixative, and refrigerated at 4°C for 24 h. For transmission electron microscope (TEM) study, the intestine was cut into small pieces (1-2 mm) in a petri dish. The tissues were rinsed with 0.1 M cacodylate buffer

(pH 7.4) 3 times (30 min each), and post-fixed in 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for 2 h. After osmication the tissues were washed 2 times (15 min each) with 0.1 M cacodylate buffer (pH 7.4) and dehydrated 15 min each in a graded ethanol series as follows: 30%, 50%, 70%, 95% (2 changes), and 100% (2 changes, 20 min each).

The TEM samples were rinsed twice for 5 min each by a transitional solvent, propylene oxide, infiltrated and embedded in Maraglas® (Electron Microscope Sciences, Fort Washington, PA) as follows: 1 Maraglas® : 1 propylene oxide (overnight, 23°C); 3 Maraglas® : 1 propylene oxide (6 h, 23°C); pure Maraglas® (1 h, 23°C), and curing in an oven at 53-55°C for 24-48 h. The tissue blocks were ultrathin sectioned (60-90 nm), and stained with uranyl acetate (2% aqueous) and lead citrate for 12 and 5 minutes, respectively. The tissue sections were viewed in a JEOL 100CX II TEM (JEOL USA, Inc., Peabody, MA).

The fixed scanning electron microscope (SEM) samples were washed twice in 0.1 M sodium cacodylate buffer for 15 min each. The tissues were post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h, and were washed two times for 10 min each in sodium cacodylate buffer. The tissue samples were dehydrated 15 min each in graded ethanol series as follows: 15%, 30%, 50%, 70%, 95%, and 100%. The tissues then were critical point dried with Ladd critical point dryer (Ladd Research Industries, Inc., Burlington, VT), mounted on stubs and coated with gold in a SPI module sputter coater (SPI Supplies Division of Structure Probe, Inc., West Chester, PA). The samples were viewed in a Philips 505 SEM (Philips Electron Optics, Inc., Hillsboro, OR).

4.4: RESULTS

4.4.1: General Observations

Angelfish in the control group were in normal body condition and exhibited no morbidity or mortality. The angelfish orally inoculated with a low (5×10^6) or a high (1×10^7) number of *S. vortens* were also in normal body condition. They were inappetent for the first three days after infection, however, they regained their appetite later. On the other hand, the angelfish intraperitoneally injected with 2×10^6 *S. vortens* showed clinical signs of anorexia, isolation, weakness, laying on their sides and respiratory distress before death. During the first week after infection, 16.7% of the angelfish displayed abnormal clinical signs, and 20% of the angelfish were dead at the end of the third week. No external gross lesions were observed during this time.

4.4.2: Gross Pathological Examination

Compared to the control angelfish, there were no distinct abnormal appearances to the internal organs of the PO-infected angelfish. General appearance of the internal organs of the IP-infected angelfish was normal, with the exception of one IP-infected angelfish which had hemorrhage in the ovary. Most intestines of the infected angelfish were swollen and had more yellow fluid than normal in the middle to posterior intestine. The angelfish IP-infected with *S. vortens* had more fluid than normal in the peritoneal cavity.

4.4.3: Light Microscopic Examination

Control angelfish

Light microscopic examination of fresh impression smears revealed no flagellates in any intestinal portions of the control angelfish.

Orally infected angelfish

A large number of *Spironucleus vortens* were seen in the fresh impression smears of the intestines of the PO-infected angelfish. The organisms were found mostly in the middle to posterior intestine (Fig. 4-8), and only a few were observed in the anterior portion of angelfish orally inoculated with low numbers of the parasites. In high dose of PO-infected angelfish, the organisms were found throughout the intestinal length. The parasites actively moved toward the intestine (histotrophic), and appeared aggregated in some areas along the intestinal mucosa (Fig. 4-9). Under microscopic observation, the majority of organisms were pyriform in shape, with multi-flagella and containing many granules inside the cells (Fig. 4-10, 4-11). There were no organisms observed in fresh smears of either blood or bile.

Intraperitoneally infected angelfish

In systemically infected angelfish, a large number of motile flagellated *S. vortens* were seen in fresh blood smears. There were also 50-60 parasites per microscopic field (10X) seen in the fresh smears of bile. The organisms were also found in abdominal fluid and a few organisms were seen in the blood vessels of fresh impressed gills. The motile flagellates actively moved toward the intestinal mucosa and they were found in all intestinal portions. Under microscopic observation, the organisms were similar to those observed in orally infected angelfish.

4.4.4: Histopathological Examination

There were no pathological changes in any tissues of the sham-inoculated control angelfish. Histopathological examination in PO-infected angelfish indicated mild to moderate multifocal enteritis with infiltration of lymphocytes and a moderated number of neutrophils in the mucosa and lamina propria. An increasing numbers of eosinophilic granulocytes (Fig. 4-12) and rodlet cells (Fig. 4-13) were observed in the submucosa and mucosa, respectively. A large number of *S. vortens* were found in all intestinal portions (anterior, middle, and posterior) throughout the experimental period (4 weeks). The organisms were usually pyriform in shape, with two sausage-shaped nuclei located in the anterior third of the cells (Fig. 4-14). The organisms also had anterior and posterior flagella, and non-staining cytoplasmic structures resembling vacuoles (Fig. 4-15). The mucosal epithelium appeared normal although the parasites were situated closely, and occasionally attached to the intestinal mucosa. However, there were no parasites or cysts observed within the intestinal tissues. Compared to control fish, histopathological findings of internal organs (spleen, liver, kidney, testis and ovary) of the PO-infected angelfish indicated normal tissue structure, and no parasites were found in these tissues.

Histopathologic examination of IP-infected angelfish revealed mild to marked granulomatous inflammation in a wide variety of internal tissues (Table 4-1). Pathological changes in the intestines of IP-infected angelfish included mild to moderate multifocal enteritis of all intestinal portions during the experimental period (4 weeks). Changes in internal organs (liver, spleen and kidney) on D7 after infection included infiltration of mainly lymphocytes and plasma cells with moderate number of neutrophils. Macrophages were observed in higher numbers in affected tissues from D14 post infection. Pathological

changes in the kidney of IP-infected angelfish exhibiting clinical disease were prominent during the second week of infection. Within the abdominal cavity, focal to diffuse infiltrates of primarily macrophages, lymphocytes and plasma cells, with fewer neutrophils, were present within the peripancreatic mesentery and serosal surfaces of the intestines (peritonitis). Fibroblast proliferation within the mesentery was consistent with the subacute to chronic nature of the lesions (Fig. 4-16). Large numbers of round to oval-shaped *Spironucleus vortens* were found within the abdominal cavity (Fig. 4-17), often associated with areas of inflammation. Occasionally, *S. vortens* were observed in mesenteric blood vessels (Fig. 4-18).

Pathological changes within the gastrointestinal tract of IP *S. vortens*-infected fish (compared to sham-inoculated controls) were similar to those observed in PO-infected angelfish. They included mild to moderate multifocal enteritis, and increased numbers of rodlet cells which were situated in the intestinal mucosa proximate to the lumen. Increased numbers of eosinophilic granulocytes were frequently present within the intestinal submucosa (Fig. 4-19), whereas moderate numbers of lymphocytes were often found within the lamina propria and basal region of the mucosa. Despite this inflammation, the mucosal epithelium appeared undamaged; there were no mucosal erosions or ulcers, and McKnight cells (apoptotic enterocytes) did not appear increased in number. All experimentally infected angelfish harbored *S. vortens* in all portions of the intestine, although the largest concentrations were in the middle to posterior segments (Fig. 4-20). Most of the intra-luminal parasites were located adjacent to the intestinal mucosa (Fig. 4-21), however, some appeared to be attached to the epithelial cells (Fig. 4-22). *Spironucleus vortens* were additionally observed within the gastric submucosa of an IP-infected angelfish (Fig4-23). They were also present in the

lumen of the stomach. Cyst forms of the parasite were not evident in any gastrointestinal site.

In one angelfish, the interfollicular areas of the ovary exhibited marked granulomatous inflammation (macrophages, lymphocytes, and plasma cells), hemorrhage, and focal areas of individual cell necrosis (Fig. 4-24). Although much of this reaction was focused upon yolk released from ruptured follicles (Fig. 4-25), the presence of *S. vortens* within macrophages (Fig. 4-26) suggests that at least part of inflammation was a response to the presence of the parasites. Oval or pyriform-shaped *S. vortens* were abundant within the ovarium interstitium and also within the periphery of ruptured follicles (Fig. 4-27). The parasites were also observed within the blood vessel in the interfollicular interstitium of the ovary (Fig. 4-28).

The most prominent histopathologic lesion in the posterior kidney was extensive mineral deposition within renal tubules (moderate to marked multifocal nephrocalcinosis), accompanied by reactive granuloma formation (Fig. 4-29). Many renal tubules were dilated, and changes within tubular epithelia included degeneration (vacuolization, spongiosis), necrosis (karyorrhexis, pyknosis, cytoplasmic fragmentation, exfoliation), and reactive hyperplasia/hypertrophy (cytomegaly and karyomegaly) (Fig. 4-30). Common glomerular alterations included the presence of increased mesangial matrix, and multiple adhesions (synechia) of glomerular tufts to Bowman's capsule. In occasional renal corpuscles, the glomerular capillaries and Bowman's space were obliterated by proliferating fibroblast-like cells (glomerulosclerosis) (Fig. 4-31). *Spironucleus vortens* were not specifically observed within the renal parenchyma. In the hepatopancreas of *S. vortens*-inoculated fish, mild mononuclear cell inflammation was exclusively associated with the exocrine pancreatic tissue. Decreased hepatocyte vacuolization (compared to control fish) was consistent with

relative fat and glycogen depletion (Fig. 4-32, 4-33). Changes within the spleen included irregular dilation of sinusoidal spaces (telangiectasis), increased numbers of eosinophilic granulocytes, and mild fibrosis at the perimeter of melano-macrophage centers (Fig. 4-34). *Spironucleus vortens* were observed within the spleen (Fig. 4-35).

4.4.5: Electron Microscopic Examination

TEM showed normal structures of brush borders, microvilli, and epithelial cells in most areas of PO- and IP-infected angelfish intestines. However, an extensive erosion of the epithelium with long stretched mitochondria inside cells was observed in one area. No parasites were found in the eroded area or within other sites of the intestinal tract. Ultrastructure and morphology of *S. vortens* has been previously described by Poynton et al. (1995). The organism was a unicellular protozoan with two sausage-shaped nuclei. Other ultrastructures, kinetosome, anterior flagellum, and posterior recurrent flagellum, were shown in Fig. 4-36. The SEM showed that parasites had anterior flagella, posterior flagella, and lateral longitudinal ridges (Fig. 4-37, 4-38) The organisms appeared attached to the intestinal mucosa in some areas (Fig. 4-39, 4-40), however, no evidence of parasites invading the intestinal epithelium was observed from the tissue samples.

4.5: DISCUSSION

This study is focused on the pathogenesis of spironucleosis in angelfish infected with *S. vortens*. Diplomonad hexamitids have been reported causing enteritis, and they have been found in many organs of angelfish (Ferguson and Moccia, 1980; Molnár, 1974). To mimic an infection occurring in nature, angelfish in the present study were orally infected with

S. vortens at 5 x 10^6 or 1 x 10^7 organisms per angelfish. To simulate systemic spironucleosis in fish, another group of angelfish was intraperitoneally inoculated with 2 x 10^6 *S. vortens* cells per angelfish in order to study the dissemination of this organism within the angelfish.

Fishes infected by diplomonad flagellates shows clinical signs varying from no clinical signs (Mo et al., 1990) to severe symptoms (Kent et al., 1992). It has been reported that infected angelfish showed no symptoms of disease although they harbored abundant numbers of amphibian *Spironucleus elegans* in the posterior part of their intestines (Kulda and Lom, 1964b). In the present study, angelfish orally inoculated with a low number (5 x 10^6) of *S. vortens* were inappetent for three days after infection, with no other clinical signs. Angelfish infected with high numbers (1 x 10^7) of *S. vortens* gained appetite later, although they were inappetent a few days longer than those infected with a low number of parasites. This suggests that the number of inoculated *S. vortens* affects duration of inappetence in infected host.

In addition to dosage (parasite number), route of infection is another factor inducing the progression of disease. The angelfish intraperitoneally injected with a lower number (2 x 10^6) of the organisms were sick, and died within three weeks after infection. The angelfish having severe systemic infection showed clinical signs of inappetite, weakness, respiratory distress, and laying on their sides. There were no external lesions observed in any of the angelfish. These results suggest that natural infection via ingestion of *S. vortens* resulting in disease is relatively uncommon regardless of the dosage of infection. The disease may become latent for long periods of time when the fish are orally infected with a low number of parasites. However, the parasites could facilitate the disease and become lethal to the host if they reach a hematogenous route.

It is believed that in systemic spironucleosis, the organisms invade the intestinal epithelium and disseminate to other tissues when the host's resistance has been suppressed (Woo and Poynton, 1995). In addition, the normally commensal organism can be pathogenic under various stressful conditions such as starvation, poor water quality, crowding, and infection from other pathogens or parasites (Specht et al., 1989; Uzmann et al., 1965). Systemic infection of hexamitids has been reported in various fish species (Mo et al., 1990; Molnár, 1974; O'Brien et al., 1993; Poppe et al., 1992; Sterud et al., 1998). In Siamese fighting fish, hexamitids were reported causing systemic infection, and the parasites were found in the abdominal cavity and in other organs such as liver, spleen, and kidney (Ferguson and Moccia, 1980). The present study revealed that *S. vortens* is able to rapidly disseminate to other internal organs and cause pathological changes once they enter the abdominal cavity. In Atlantic salmon, *Spironucleus sp.* causing a similar systemic infection were found in all internal organs (Poppe et al., 1992). In the present study, no parasites were observed in the kidney or liver of the angelfish. In IP-infected angelfish, the organisms were occasionally observed in the gastric submucosa which indicates an ability of the organism to invade from the abdominal cavity into the gastric submucosa. However, the parasites were not observed in the mucosal layer or at the serosa of the intestine. It is suggested that *S. vortens* in IP-infected angelfish disseminated to other organs via blood circulation, and reached the intestines after they were released into the bile.

Transmission of hexamitids is believed to occur via ingestion of cysts and/or trophozoites contaminated in the environment (Lom and Dyková, 1992). To date, cysts of fish hexamitids are very rare either in cell culture or in host feces. They have been reported in an early study of *Octomitus salmonis* in trout (Moore, 1922), but were not detected in recent studies of the same organism (Kent et al., 1992; Kulda and Lom, 1964a; Tojo and Santamarina, 1998).

Although cysts of *Hexamita sp.* have been observed in the intestinal epithelium of infected fish (Sano, 1970), cysts of *S. vortens* were not found in any tissues or in the intestinal lumen of the angelfish in this study. This supports the suggestion that trophozoites of *Spironucleus* play a role in transmission from one fish to another by contamination through the environment (Sterud et al., 1998). Diffuse granulomatous inflammation is the marked pathological changes found in the internal organs of the IP-infected angelfish. In the present study, tissue reaction (diffuse granulomatous inflammation) in the internal organs, and mild enteritis with infiltration of mononuclear in the lamina propria of the intestines were similar to the pathogenesis of systemic spironucleosis in angelfish reported by O'Brien et al. (1993). Large numbers of *S. vortens* were observed in the angelfish's ovary with an extensive infiltration of various inflammatory cells. This indicates that systemic spironucleosis may directly affect the reproductive performance of angelfish. The pathological changes associated with the kidney mostly involved in renal tubules and glomeruli, although no parasites were located in these areas. Decreasing vacuolization was observed in the hepatopancreas of IP-infected angelfish suggesting a state of fat and glycogen depletion of hepatocytes, possibly indicating a condition of malnutrition of the host. Therefore, it is suggested that systemic spironucleosis causes a wide range of tissue reactions in the internal organs, and either the organism or products (toxins and enzymes) released from the organisms may induce the tissue reactions in those organs.

The present study suggests that *S. vortens* normally are commensal organisms in the intestine where they may cause only mild enteritis with no morbidity and mortality. Any factors lowering the host immune response may provoke the commensal *Spironucleus vortens* to become a pathogenic organism (Lom and Dyková, 1992). The parasites can then become lethal to the host once they reach the abdominal cavity or enter the blood circulation.

Diffuse granulomatous inflammation with infiltration of lymphocytes, macrophages, and plasma cells is the major tissue reaction observed in most internal organs of *S. vortens*-infected angelfish. The parasites may disseminate to other organs via the blood circulation after the organism invades the mesenteric blood vessels. However, the mechanism by which the organism invades blood vessels or ability of the organism to directly invade the internal organs is still unknown.

4.6: ACKNOWLEDGMENTS

I would like to thank Dr. John L. Robertson and Dr. Jeffrey Wolf for help in interpreting the tissue samples in the histopathological study. I thank Ms. Ginny Viers and Ms. Kathy Lowe for help in tissue preparation for electron microscopic examination and Dr. Caceci for assistance with the EM studies. I also thank Korawuth Punareewattana and Amornchai Somjetlertcharoen for help in sample collections.

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CHAPTER 5

IMMUNE RESPONSE OF ANGELFISH INFECTED WITH *SPIRONUCLEUS VORTENS*

5.1: ABSTRACT

Fish immunity against hexamitid flagellates was studied in angelfish infected with *Spironucleus vortens*. Cell-mediated immunity was examined by the presence of responding cells (inflammatory cells) infiltrating into the affected tissues, and from the abilities of kidney macrophages to increase production of hydrogen peroxide (H_2O_2), and to phagocytose microspheres. Anti-*S. vortens* humoral antibodies in infected fish sera were evaluated by a gel immunodiffusion method. Histopathological study revealed that macrophages in the infected tissues where the organisms lived *in situ* were activated, and possibly played a role in eliminating the parasites. Results of the *in vitro* studies of *S. vortens*-infected angelfish indicated that kidney macrophages did not produce a significant increase in H_2O_2 production or engulf more microspheres than the control fish. Other inflammatory cells responding to the infection of *S. vortens* were lymphocytes, eosinophilic granulocytes, neutrophils, and plasma cells. Rodlet cell located in the intestinal mucosa responded to the infection by increasing in cell number, however, its role is still unknown. Results of the humoral immune response evaluation indicated that there were no anti-*S. vortens* specific antibodies detected from sera of the infected angelfish. This study suggests that cell-mediated immunity, especially the localized response from the inflammatory cells, is important in eliminating a *Spironucleus vortens* infection in angelfish.

5.2: INTRODUCTION

The fish's immune response, like that of many vertebrates, is an important defense system in maintaining the integrity of self and protecting the host from pathogens. Natural or innate immunity is the first defense mechanism protecting a host from invading organisms, while acquired immunity is developed later after an infection (Woo, 1996). Acquired immunity is generally more intense in controlling and eliminating infections. There have been several studies examining the function of acquired immunity against organisms through humoral (Jones et al., 1993; Lin et al., 1996; Plumb, 1973) and cell-mediated (Avtalion and Shahrabani, 1975; Sharp and Secombes, 1993) immune responses.

Humoral immunity, antibody, has been shown to provide protection to fish from reinfections of the parasitic flagellate, *Amyloodinium ocellatum.* Tilapia recovering from an infection of dinoflagellates developed specific antibody which agglutinated and killed the parasites in cell culture (Smith et al., 1993). This antibody in hybrid striped bass was higher in both infected fish in culture and the experimentally immunized fish than in uninfected fish (Smith et al., 1994). Antibody in the plasma of goldfish has been shown to protect another group of goldfish receiving immune plasma from the infection of blood parasites, *Trypanosoma sp.* (Woo, 1981). The antibody not only protects against one species of the organism, but also may cross protect against other species of parasite (Wolf and Markiw, 1982).

Cell-mediated immunity (CMI) is another defense system used to protect the host from invading organisms. Phagocytes are major immune cells of CMI functioning in recognition and elimination of invading organisms and damaged tissue. The predominant phagocytic

cells in fish are the granulocytes (neutrophils) and mononuclear cells (macrophages and monocytes) (Ainsworth, 1992; Secombes and Fletcher, 1992). Neutrophils are the first cell type entering the inflammatory area, and respond by secreting reactive oxygen species such as superoxide anion, singlet oxygen, hydrogen peroxide, and hydroxyl radicals (Rice et al., 1996). Monocytes and tissue macrophages are other cell types functioning in phagocytosis and secretion of reactive oxygen species, and as a proinflammatory cell secreting cytokines (Rice et al., 1996). The killing activity of fish macrophages, like that of mammalian phagocytes, is largely dependent on the respiratory burst following phagocytosis (Secombes and Fletcher, 1992). During the respiratory burst, several reactive oxygen species are produced, therefore many different assays have been developed to measure the free radicals. A singlet oxygen is one of free radicals produced from the reaction, and it emits light in the form of chemiluminescence which can be detected in flow cytometry (Secombes and Fletcher, 1992).

In fish, parasitic flagellates are commonly found on the gills and body surface, in the digestive tract, and in the blood and tissue fluids (Woo, 1996). Most immunological studies of fish flagellate have been concerned with external parasitic flagellates and blood parasitic flagellates, surprisingly none have studied the intestinal flagellates including the diplomonad flagellates (hexamitids). The present study investigated the immune response of the freshwater angelfish (*Pterophyllum scalare*) infected with *Spironucleus vortens*. Humoral immunity (antibody) to the parasites was evaluated by precipitation reaction in agarose gels. Cell-mediated immunity to *S. vortens* was examined in activated macrophages through the production of hydrogen peroxide (H_2O_2) and phagocytosis.

5.3: METHODS

5.3.1: Fish

Adult angelfish (120 fish) with an average weight of 8.74 ± 2.19 g were raised in 20-gallon tanks (15 fish/tank), with a biofilter system, heater, and aerated circulation. The angelfish were divided into a control and 3 experimental groups with 30 fish in each group. All fish were kept in the same room throughout the experiment at a temperature of 28°C and a 14/10 hour light/dark photoperiod. The angelfish were fed a commercial prepared flake diet (TetraMIN®, Tetra Sales, Blacksburg, VA) twice a day.

5.3.2: Infection of *Spironucleus*

Spironucleus vortens cultivated in TYI-S-33 medium were harvested in log phase by centrifugation at 360 x g for 7 minutes. The parasites were washed 3 times with PBS (pH 7.4), resuspended in PBS, and counted by automatic cell counter (CASY®1; model TTC, Schärfe System GmbH, Germany).

The angelfish were anaesthetized by immersion in water mixed with equal amounts of MS-222 and sodium bicarbonate ($NaHCO_3$). Using a micropipette tips attached with a small plastic tube, 60 angelfish (30 fish/experimental group) were orally inoculated with either 5 x 10^6 (PO1) or 1 x 10^7 (PO2) cells of *S. vortens*. Another 30 angelfish were intraperitoneally inoculated with 2 x 10^6 (IP) cells of *S. vortens*. On the same day, 30 angelfish in two control groups (15 fish each) were either orally gavaged or intraperitoneally injected with PBS (pH 7.4) in the same manner as those of the experimental groups. After inoculation or

gavaging, fish were immediately returned to the water and observed for any abnormal signs induced by the inoculation method.

5.3.3: Serum Collection

Control serum was collected from 7 angelfish before inoculation on the first day (d1) of experiment. On d7, d14, d21 and d28, 6 fish from each group of fish were anaesthetized with MS-222, and blood of the angelfish was withdrawn from a heart using an insulin syringe fitted with a 28 gauge needle (Fig. 5-1, 5-2). The blood was allowed to clot at room temperature for 1 h. Serum was separated by centrifugation at 14,000 x g for 5 min in pediatric centrifuge tubes (Microtainer, Becton Dickinson and Co., Rutherford, NJ) and stored at -80°C until use.

5.3.4: *Spironucleus* Antigen Preparation

Log-phased *S. vortens* grown in TYI-S-33 medium were harvested by centrifugation at 360 x g for 7 min. The organisms were washed 3 times in 3 ml of PBS (pH 7.4), counted in a hemacytometer chamber, and resuspended in 1 ml of PBS, pH 7.4. The organisms (1.2 x 10^7 cells ml^{-1}) were disrupted in a 12 x 75 mm tube (on ice) 2 times for 15 sec on- and off-cycles with an ultrasonic cell disrupter (Sonar Dismembrator, model 300, Fisher Scientific, Pittsburgh, PA) at 35% capacity. The suspension was centrifuged at 60 x g for 5 min at 4°C, and the supernatant was transferred into an eppendorf tube. The *Spironucleus vortens* soluble antigen was kept at -80°C until use.

5.3.5: Collection of Kidney and Preparation of Cell Suspensions

After serum collection, the anaesthetized angelfish of the control and experimental groups were killed by cervical separation and cut along the abdominal wall to reveal the internal organs. Kidneys were carefully removed from fascia attached to them, and then were immediately placed in 2 ml of sterile RPMI-1640 culture medium (Mediatech Inc., Herndon, VA). Cells in a kidney were dissociated in 2 ml RPMI by gently abrading the tissue on a 60-mesh stainless steel sieve screen. Cells were harvested by centrifugation at 360 x g for 7 min at room temperature (23°C), washed 3 times in 2 ml PBS, and resuspended in 1 ml PBS, pH 7.4. Quantification of cell suspension was done by automatic cell counter (CASY®1; model TTC, Schärfe System GmbH, Germany). All cell suspensions were standardized to the same final concentration of 1.0×10^6 cells ml^{-1} for the study of cell-mediated immune response.

5.3.6: Cell-Mediated Immunity (CMI)

Cell-mediated immunity (CMI) against *Spironucleus vortens* infection was investigated in two studies;

1) localized leucocyte response, and
2) macrophage function test
    - 2.1) chemiluminescence assay and flow cytometric analysis
    - 2.2) phagocytosis of fluorescent microspheres.

5.3.6.1: Localized Leucocyte Response

Histopathological sections of angelfish intestines and internal organs were examined under a compound microscope for the response of inflammatory cells. The reacting cells (leucocytes, macrophages, and plasma cells) at the invaded sites or sites associated with the infection were compared between control and infected fish. Types of infiltrating cells were examined and recorded.

5.3.6.2: Macrophage Function Test

5.3.6.2.1: Chemiluminescence Assay and Flow Cytometric Analysis

Kidney macrophages prepared as previously described (section 5.3.5) were evaluated in a flow cytometer for a production of hydrogen peroxide (H_2O_2). The leucocytes (macrophages) in suspensions were adjusted to 1.0×10^6 cells ml^{-1} in PBS, pH 7.4. Aliquots containing 250 µl of macrophages (0.25×10^6 cells) were first incubated with 5 µl dichlorofluorescin diacetate (DCF-DA; Sigma, St. Louis, MO; 5 mM) at room temperature (23°C) for 15 minutes. The cell suspensions then were incubated with 10 µl phorbol-12 myristate 13-acetate (PMA; Sigma, St. Louis, MO; 100 ng ml^{-1}) at room temperature (23°C) for 30 minutes. At the end of the incubation period, cells were immediately placed on ice and evaluated by flow cytometry. Cells were analyzed using a Coulter Epics XL flow cytometer (Beckmann Coulter, Hialeah, FL). The control cell suspension was prepared as described. A population of large cells containing phagocytic cells was electronically isolated on a two-parameter histogram of forward angle light scatter (FALS) and side angle light scatter (SALS). For each sample, 5000 events of large cells with high granularity were collected and analyzed.

5.3.6.2.2: Phagocytosis Assay and Flow Cytometric Analysis

Kidney macrophages (section 5.3.5) were adjusted to 1.0 x 10^6 cells ml^{-1}. Aliquots containing 500 µl of macrophages (0.5 x 10^6 cells) were added with 45 µl of diluted (100 beads $cell^{-1}$) carboxylate-modified microspheres (FluoSpheres®, Molecular Probes Inc., Eugene OR; 2.0 µm, No. F-8827). The volume of cell suspension then was raised to 4.0 ml by RPMI supplemented with 100 IU penicillin-streptomycin (Fisher Scientific, Pittsburgh, PA, No. MT30001CI), 10% non-essential amino acid (Mediatech Inc., Herndon, VA, No. 25-025-CI), and 10% of complement-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA, No. S11510). The culture tubes were capped loosely, and placed in an incubator at 23°C (room temperature) with 5% CO_2 for 18 h. After the incubation period, the cell suspensions were removed from the incubator and washed twice using RPMI. The cells were resuspended in 0.5 ml RPMI, and immediately evaluated by flow cytometry as previously described. For each sample, 5000 events of large cells with high granularity were collected and analyzed.

5.3.7: Humoral Immunity

5.3.7.1: Gel Diffusion Precipitation (Ouchterlony) Test

The presence of antibody to *S. vortens* antigens was determined by the Ouchterlony gel diffusion method. Agarose (1%) in tris-tricine buffer was prepared and used as medium for carrying both antigen and antibody. The liquefied agarose (8 ml) was spreaded on a piece of Gelbond film® placed and fitted on a 75 x 50 mm glass slide. When the agar had completely solidified, a template was placed over the gel, and the gel was cut with an agar

punch. To properly identify orientation, each gel was cut at the upper left corner, and the number of the gel was assigned at this corner. After removing the agarose plug, 25 µl of serum to be tested was added into designated outer wells and 25 µl sonicated *Spironucleus* antigens placed in the center well. The glass slides with gels were placed in a moisture chamber and incubated at room temperature. The materials in the gels were allowed to diffuse overnight and the precipitin lines were examined after 24 h. To keep gels as permanent records, gels were washed in phosphate buffed saline (PBS) which was changed once a day for 2 days. Gels were stained in Coomassie Brilliant Blue (G-250) for 10 minutes and destained in a mixed solution of 0.45% methanol and 0.1% glacial acetic acid. Gels were then dried in a 37°C incubator overnight and kept as a permanent record.

5.3.8: Statistical Analysis

Percentages of macrophages producing H_2O_2 and expressing phagocytosis were analyzed and compared by a SAS statistic program (SAS Institute Inc., Cary, NC). Percentages of macrophages expressing phagocytosis were transformed to natural logarithmic values in order to stabilize the variances for analysis purpose. The percentages of macrophages were analyzed and compared by ANOVA test at $\alpha = 0.05$.

5.4: RESULTS

5.4.1: Localized Leucocyte Response

Inflammatory cells responding to the infection of *S. vortens* were most notable in the IP-infected angelfish and have been described in the previous study (Chapter 4). Microscopic

examination revealed focal to diffuse infiltration of inflammatory cells in the affected tissues. Macrophages, lymphocytes and plasma cells were the primary infiltrating cells found within the abdominal cavity. These responding cells were located within the peritoneum lining in response to the parasites (Fig. 5-3). They also infiltrated into the peripancreatic mesentery and intestinal serosa. Pathological changes within the intestines were mild to moderate multifocal enteritis. Lymphocytes and a moderated number of neutrophils were associated with the enteritis, and were often present within the lamina propria and basal region of the mucosa. Eosinophilic granulocytes were frequently found in the intestinal submucosa at a higher number than in the normal control intestine. Rodlet cells were another cell type often found in increased numbers in the intestinal mucosa of infected angelfish.

Diffuse granulomatous inflammation was the main pathological changes observed in the internal organs (ovary, kidney, liver and spleen) of the infected angelfish. Macrophages, lymphocytes, and plasma cells infiltrated most of the interfollicular areas of the ovary. Yolk material, dead host cells, and parasites were frequently found inside the macrophages in this area (Fig. 5-4). The major pathological changes in the kidney were associated with an increased deposition of mineral and an increased inflammatory response. Large numbers of mononuclear cells (mostly lymphocytes and macrophages) infiltrated into the kidney parenchyma, and a moderated number of plasma cells were also observed in the same locations. Mild inflammation was also observed in the liver. Lymphocytes were evenly disseminated in most parts of the liver parenchyma while a small number of macrophages were occasionally found in some areas. Plasma cells were mostly located inside the blood vessels in the pancreatic areas. In the spleen, an increased number of eosinophilic granulocytes was observed in the parenchyma. Other inflammatory cells, i.e. leucocytes and macrophages, also infiltrated into areas where the parasites were located.

5.4.2: Macrophage Function Test

5.4.2.1: Chemiluminescence Assay and Flow Cytometric Analysis

Kidney macrophages of infected angelfish were examined for a production of hydrogen peroxide (H_2O_2) after they had been incubated with DCF-DA and activated by PMA. The proportion of cells expressing chemiluminescence were electronically measured in a flow cytometer as shown in Fig. 5-5. Percentages of macrophages having chemiluminescence on each testing date were shown in Fig. 5-6, Fig. 5-7, and Table 5-1. The results indicated that there was no difference in numbers of macrophages producing H_2O_2 between control and experimental groups. There was also no difference in numbers of cells producing H_2O_2 between PO- and IP-infected angelfish. However, macrophages of the angelfish orally infected with a low number (5×10^6) of *S. vortens* (PO1) had a trend toward an increase in H_2O_2 production after d14, while in angelfish orally infected with a high number (1×10^7) of *S. vortens* (PO2) displayed a trend toward a decrease in H_2O_2 production after the infection. The production of H_2O_2 was also increased in IP-infected angelfish after d14 as observed in the PO1-infected angelfish.

5.4.2.2: Phagocytosis Assay and Flow Cytometric Analysis

The ability of kidney macrophages of *S. vortens*-infected angelfish to engulf fluoresbrite microspheres was electronically measured by a flow cytometer as shown in Fig. 5-8. Percentages of large granular cells (macrophages) expressing fluorescence inside the cells between control and infected angelfish were analyzed and compared. The results of phagocytic cells is shown in Fig. 5-9, Fig. 5-10, and Table 5.2. There was no significant difference between the percentages of phagocytic cells dissociated from angelfish PO- or

IP-infected angelfish, regardless of dosage or route of administration. There was also no difference between the phagocytic cells of infected and non-infected angelfish. The phagocytic cells of all angelfish had a reduced ability to engulf the fluoresbrite microspheres from d7 of an infection.

5.4.3: Humoral Immunity

The antigen-antibody interaction on the agar gels resulted in no precipitin line between *S. vortens* antigens and all angelfish sera. Therefore, no anti-*S. vortens* antibodies were detected in any of the angelfish sera.

5.5: DISCUSSION

Histopathological examination revealed that most of the *S. vortens* organisms were located in the intestinal lumen; some possibly attached to the intestinal mucosa. Moderate number of lymphocytes and neutrophils infiltrated the mucosa, but no parasites were found in this area. There did not appear to be a severe inflammatory reaction damaging to the mucosal tissue caused by these two cell types. The inflammatory reaction was more intensive in angelfish harboring *S. vortens* in the submucosa. The tissue reactions in the submucosa included blood vessel congestion, tissue edema, and infiltration of inflammatory cells, especially eosinophilic granulocytes. This suggests that the tissue reactions were a host response for eliminating the organisms. Tissue inflammatory reaction helped increase the blood supply to the affected tissues resulting in an increased attracting and infiltrating of the inflammatory cells into those areas. Tissue edema was another response of the host to help eliminate the parasites or to confine the organisms and prevent dissemination into other

areas. Eosinophilic granulocytes are motile phagocytic cells that can migrate from the blood into the tissue space, and they have often been found in the fish response to parasitic infestations. In carp (*Cyprinus carpio*), eosinophilic granular cells were the predominant cells infiltrating tissue sites invaded by *Ichthyophthirius multifiliis* (Cross and Matthews, 1993). This cell type was also found mostly in the intestinal submucosa of angelfish infected with *S. vortens*. The contents inside granules of eosinophilic granular cells may damage the parasite membrane (Kuby, 1994). However, there was no evidence of cell-mediated damage to the *Spironucleus vortens* in the present study. An increased number of rodlet cells were also found within the mucosa of infected angelfish. It has been suggested that rodlet cells are one type of granulocyte (Smith et al., 1994). In the present study, an increased number of rodlet cells were associated with the infection of *S. vortens*, however, the functions of this cell type is still unknown.

In *S. vortens*-infected angelfish, focal to diffuse granulomatous inflammation was the primary pathological change observed in the internal organs of the abdominal cavity. Macrophages, lymphocytes, and plasma cells were the most common inflammatory cell type infiltrating into the affected tissues. The roles of macrophages, lymphocytes, and plasma cells in the fish immune response include phagocytosis, enhancing the inflammatory response, and antibody production, respectively (Kuby, 1994). In the infected ovary, macrophages were actively phagocytic engulfing whole *Spironucleus vortens*, yolk material, and dead host cells. The presence of lymphocytes in the tissues suggested that they help activating and/or enhancing the phagocytosis of macrophages. It was noticed that the inflammatory cells were active and found abundantly in organs (ovary, spleen, and abdominal cavity) where the parasites were also located. In contrast, the inflammatory cells were found in lesser numbers in the kidney and liver of infected angelfish.

Fish macrophages have been frequently harvested from the fish kidney in several studies of cell-mediated immune response (Buchmann and Bresciani, 1999; Graves et al., 1985; Nie et al., 1996). In the present study, macrophages of *S. vortens*-infected angelfish were also dissociated and harvested from the kidney. The results indicated that kidney macrophages of *S. vortens*-infected angelfish did not produce a significant increase in H_2O_2 production or engulf more microspheres than the control fish. There was a potential trend toward an increase in H_2O_2 production after 21 days post-infection, however, further examination of this observation needs to be conducted. In the *in vitro* chemotaxis assay, macrophages were attracted and had a slightly increase in the respiratory burst level after exposure to the parasite, *Gyrodactylus derjavini* (Buchmann and Bresciani, 1999). As previously mentioned, there were no parasites observed in the kidney, and the kidney macrophages were found in lesser number than was observed in other *S. vortens*-infected organs. Therefore, the kidney macrophages did not appear to be in close contact with, or stimulated by the parasites. This suggests that macrophages will be activated to produce H_2O_2, and perform phagocytosis only when they are in close contact with the organisms. Antigen structure, dosage, and route of administration are some factors that determine whether the response of the immune system to antigens will lead to immunity or tolerance (Kuby, 1994). Evidence of immunosuppression in a proliferation of lymphocytes has been reported in carp lymphocytes exposed to a high concentration of cestode (*Bothriocephalus acheilognathi*) extract (Nie et al., 1996). In the present study, angelfish were inoculated with 2×10^6 or 1×10^7 cells of *S. vortens*. Results of the humoral immune response revealed no anti-*S. vortens* antibodies produced in any of the infected angelfish. Although, many lymphocytes and plasma cells were found in the infected tissues, they may not be able to produce antibodies due to an immunosuppression or immunotolerance. However, it is unknown how the organisms may suppressed or caused tolerance to the immune system.

5.6: ACKNOWLEDGMENTS

I thank Korawuth Punareewattana and Amornchai Somjetlertcharoen for help with sample collections.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Freshwater angelfish (*Pterophyllum scalare*) are one of the most commercially important aquarium fish. The cost of an angelfish is not only based on size but also color and strain. Raising and breeding angelfish in a warmwater facility is now applicable for most aquaculturists. Good quality feed and water are considered important factors for fish health. However, pathogenic organisms causing diseases in fish are also considered a major factor of economic loss in the fish industry. Diplomonad flagellates are among the known pathogenic organisms causing disease and death in fish. *Spironucleus sp*. is one of hexamitids frequently reported in the angelfish. This parasite has also been reported to be a serious pathogen causing death in other cichlids.

Spironucleus vortens were examined in the present study in both *in vitro* and *in vivo* experiments. The first experiment was focused on optimal conditions *in vitro* for the growth of *S. vortens*. The second experiment examined the effectiveness of various chemotherapeutic agents on the growth of the organisms. The last two experiments investigated the intestinal pathogenesis and immune response of angelfish infected with the parasites.

The study of optimal requirements for the growth of *S. vortens* was the first report in this species. The organisms have a high growth rate at a temperature of 25°C and a pH of 6.5 to 7.5. The parasites did not require bile for growth, but could live for a short period of time in an environment with a low concentration of bile. The parasites survived longer at a lower temperature (22°C), however they were less active than those at 25°C. This suggests that low

temperature may facilitate the risk of a subclinical infection in angelfish. At higher temperature (34°C), the parasites were inhibited and killed within 24 hours. Therefore, environmental temperature is an important factor in the growth of *S. vortens*, and this information may be useful in the control and prevention of spironucleosis.

Seven chemotherapeutic agents (dimetridazole, metronidazole, pyrimethamine, magnesium sulfate, albendazole, fenbendazole, and mebendazole) were examined for their effectiveness in inhibition of the parasite growth. In this study, dimetridazole, metronidazole, and mebendazole were the most effective chemicals in inhibiting the growth of *S. vortens*. Dimetridazole is not commonly used in the fish industry as metronidazole due to its toxicity and carcinogenicity. However, the results of this study showed that dimetridazole was more effective in growth inhibition. It is interesting that mebendazole was also effective in growth inhibition of this parasite because this chemical is a broad spectrum and safe chemical widely used to treat parasitic infections in small and large animals. Therefore, the benzimidazoles may be useful chemicals in the treatment of spironucleosis and other parasitic infections in fish.

The pathogenesis of spironucleosis was studied in angelfish orally infected and intraperitoneally inoculated with *S. vortens*. The orally infected angelfish were inappetent with no other clinical signs after infection. In contrast, the IP-infected angelfish displayed inappetent, weakness, laying on their sides, and respiratory distress before death. Morbidity and mortality of IP-infected angelfish were observed in the first three weeks after infection. No external lesions were observed in any angelfish. Large numbers of the organisms were found in the intestinal lumen and in the gastric submucosa of IP-infected angelfish. However, no invading parasites were found in the intestinal mucosa. Histopathologic

examination of the gastrointestinal tract revealed a mild to moderate multifocal enteritis in infected angelfish. There were increases in rodlet cell numbers located in the intestinal mucosa, and of eosinophilic granulocytes in the intestinal submucosa. Lymphocytes and a moderate number of neutrophils were often observed within the lamina propria and basal region of intestinal mucosa. Pathological changes in the internal organs of IP-infected angelfish included mild to marked granulomatous inflammation. Macrophages, lymphocytes, and plasma cells were frequently observed in the abdominal cavity (peritonitis), within the peripancreatic mesentery, and in the interfollicular areas. The most prominent histopathologic lesion in the excretory (posterior) kidney was extensive mineral deposition within the renal tubules and glomeruli. There was a mild mononuclear cell inflammation in the hepatopancreas, and an overall decrease in the hepatocyte vacuolization indicating the stage of fat and glycogen depletion. Changes within the spleen included telangiectasis, increased numbers of eosinophilic granulocytes, and mild fibrosis at the perimeter of melano-macrophage centers. The pathological changes observed in the internal organs and intestines lead to the conclusion that *S. vortens* normally does not cause systemic spironucleosis, but could cause a mild enteritis to the host. However, they may be lethal to the host and cause systemic spironucleosis if they are able to enter the abdominal cavity and then disseminate to other organs via the blood circulation.

The immune response to hexamitids in fish is unknown. The purpose of the last part of this study was to investigate humoral and cell mediated immunity in *Spironucleus vortens*-infected angelfish. Normally, fish start developing an immune response during the second week after a parasitic infection. However, there were no anti-*S. vortens* antibodies detected from sera of infected angelfish during the first four weeks of infection. The results of assays to evaluate cell-mediated immunity indicated that macrophages derived from the

kidney were not activated to produce H_2O_2 or to engulf microspheres. However, histopathological examination showed that increased numbers of macrophages were present in the tissues where the parasite lived *in situ* (ovary and peritoneum). Therefore, it is likely that angelfish macrophages are activated only when in close contact with *S. vortens*. Method by which the parasites interact with fish macrophages is still unknown and needs further study.

This study provides information on the basic environmental requirements of the parasites, the effectiveness of chemicals for the treatment of spironucleosis, preliminary information on the pathogenesis of spironucleosis, and preliminary information on the immune response of angelfish against an infection of *S. vortens*. The life cycle of hexamitids, especially *Spironucleus,* is based on a life cycle of other diplomonad flagellates, and it is believed that cysts and trophozoites are transmitted from one host to another host. However, this author believes that trophozoites of *Spironucleus vortens* are more important than cysts in nature. The trophozoites play a role in transmission, cause pathological changes, and may be lethal to the host in the systemic infection. It is suggested that *Spironucleus vortens* can cause systemic infection only if the organisms were misplaced into the abdominal cavity or into the blood circulation. To completely understand a life cycle of *Spironucleus vortens*, we still need to study what factors favor trophozoites but limit the formation of cysts, and if cysts exist, how they affect to the host. Although the results of this study are specific to *Spironucleus vortens*, the author believes they are useful for understanding the nature of other hexamitids.

FIGURES

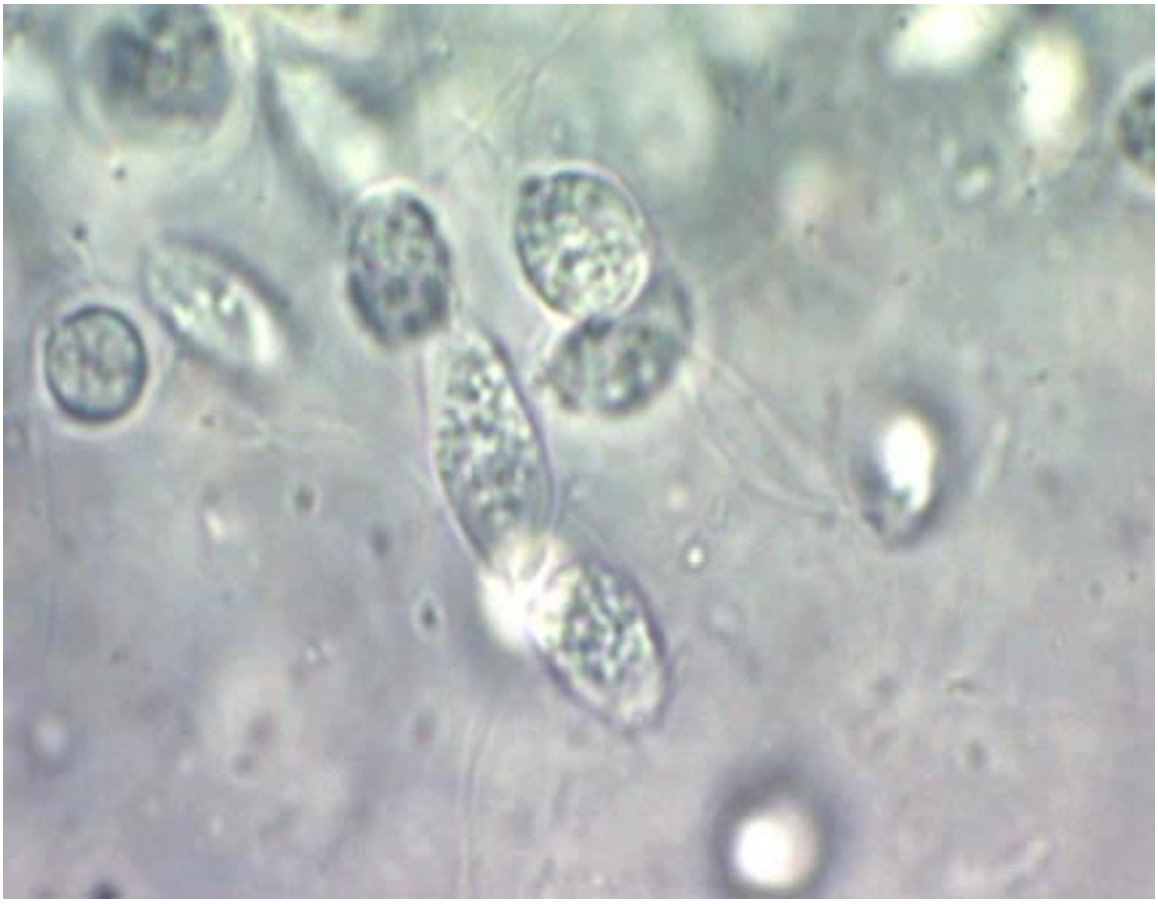

Figure 1-1. General morphology of *S. vortens* from fresh impression smear of intestine (100X).

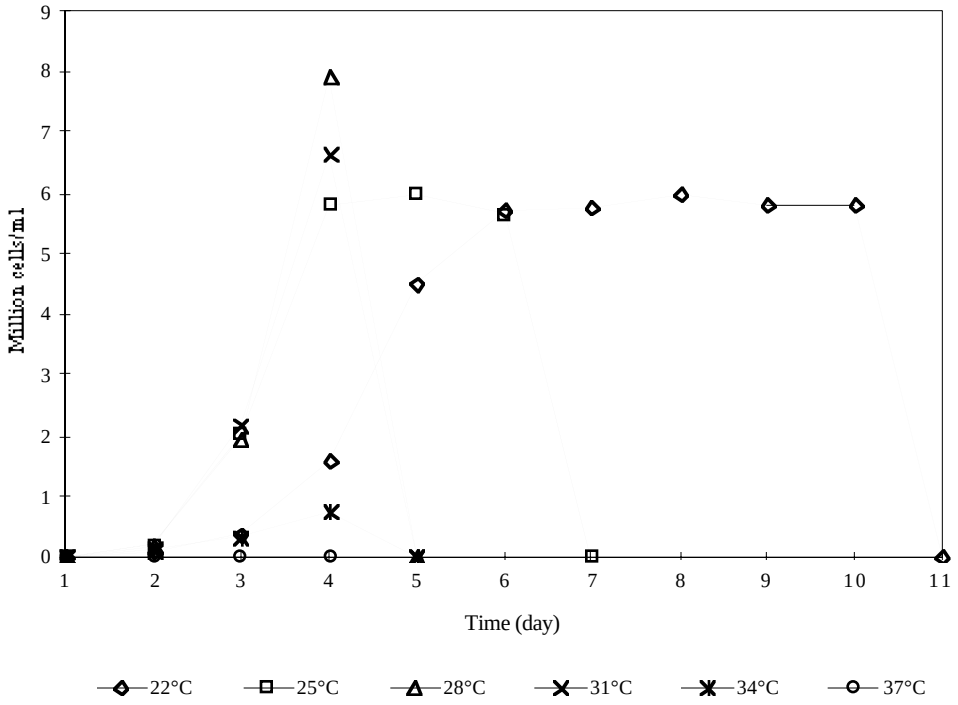

Figure 2-1. Growth of *S. vortens* cultivated under different temperatures (°C)

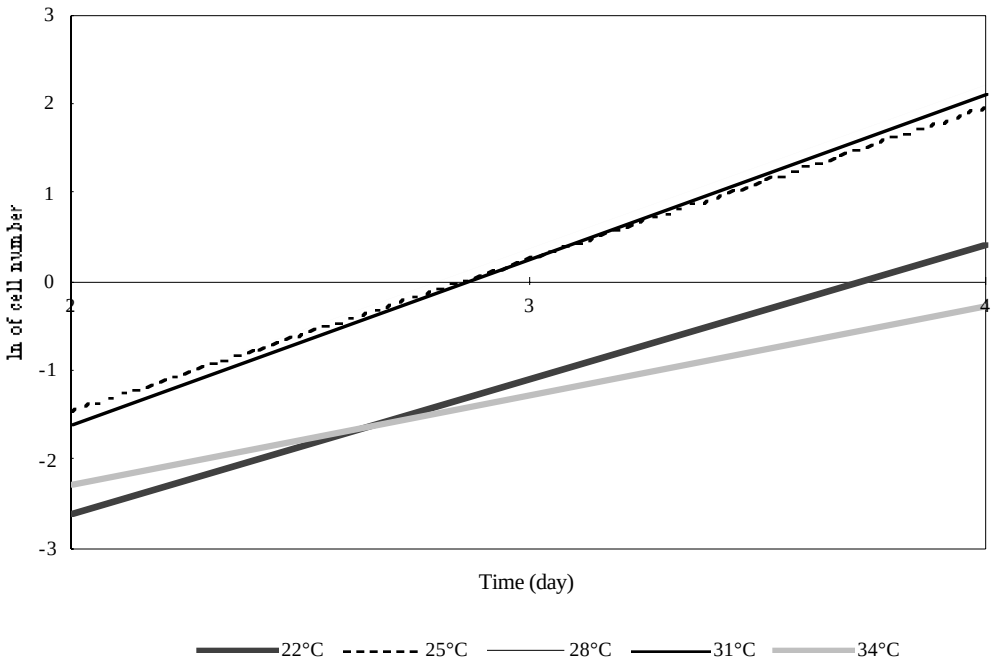

Figure 2-2. Transformed growth curves of *S. vortens* cultivated under different temperatures (°C)

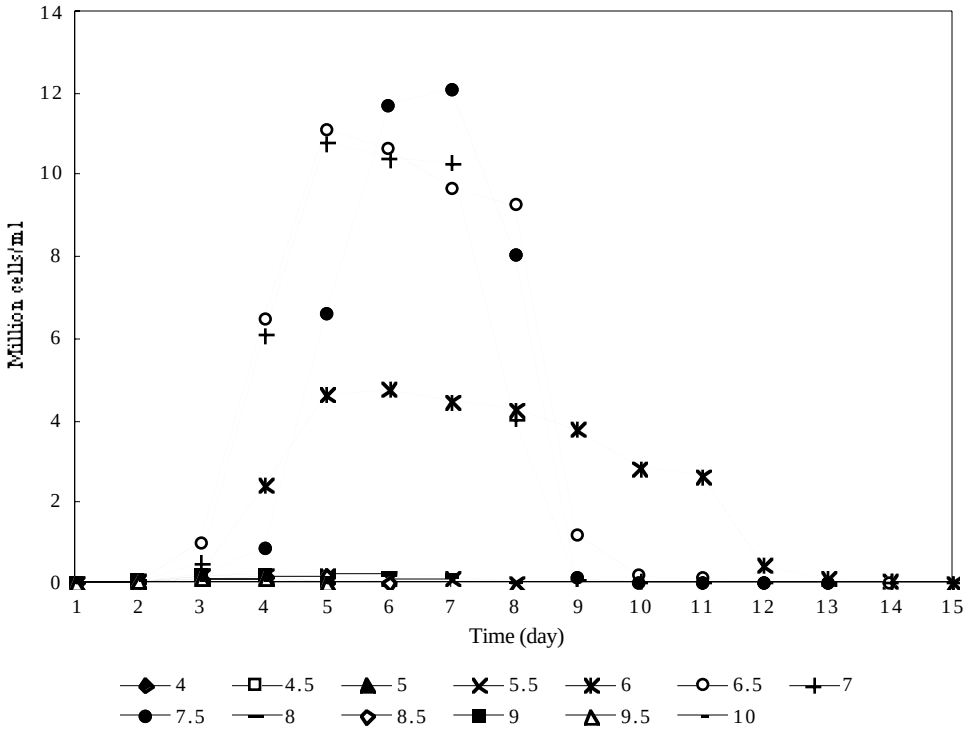

Figure 2-3. Growth of *S. vortens* cultivated under different pH conditions

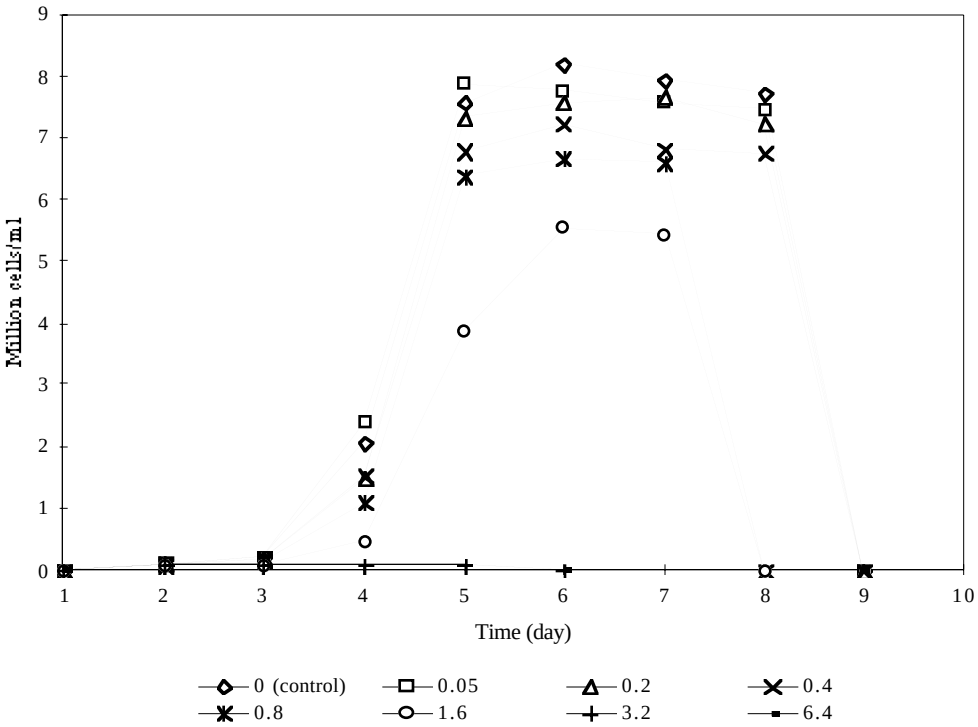

Figure 2-4. Growth of *S. vortens* cultivated in different concentrations (mg ml^{-1}) ofbovine bile

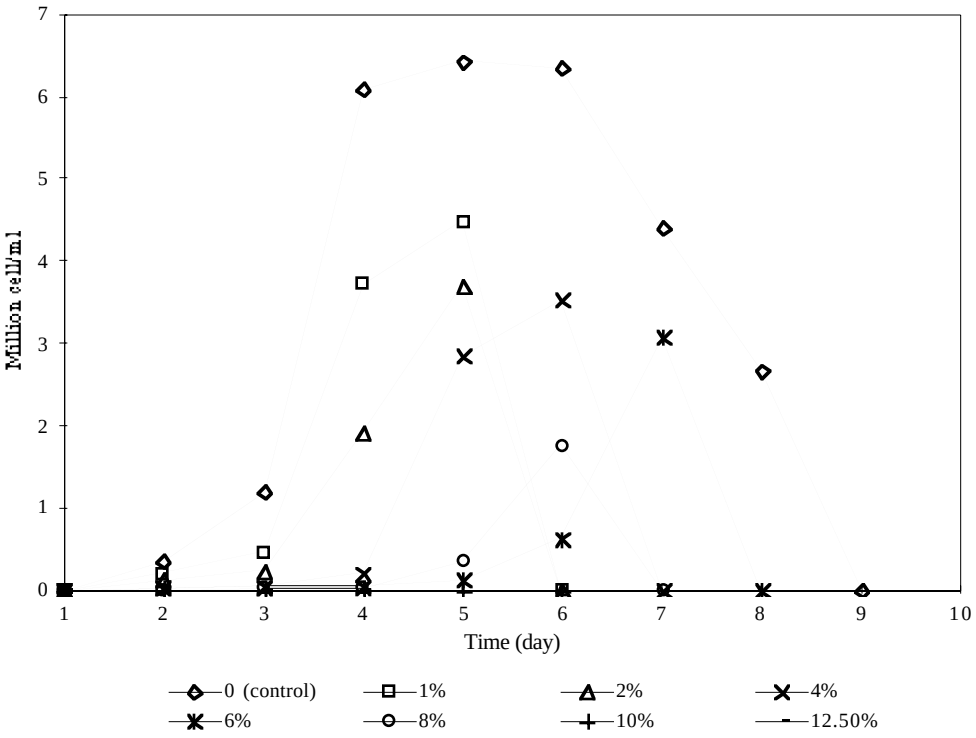

Figure 2-5. Growth of *S. vortens* cultivated in different concentrations (%) of fish bile

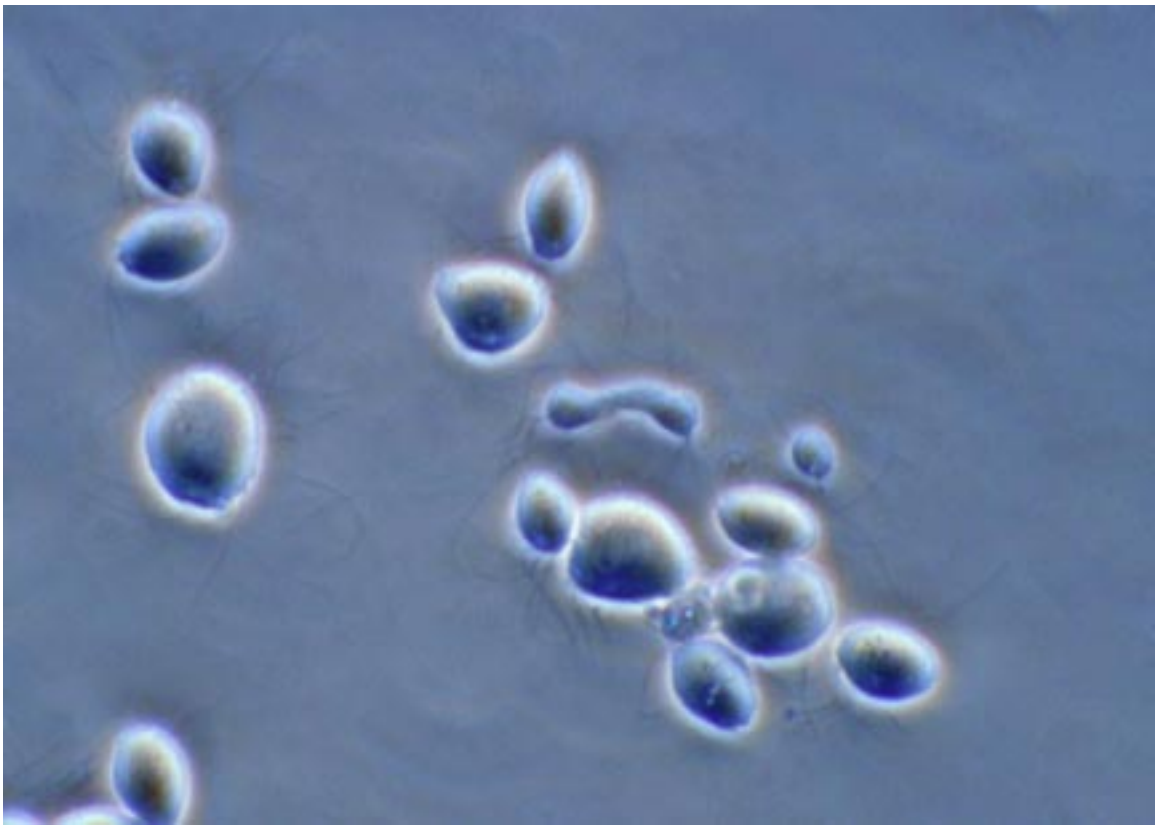

Figure 2-6. The majority of *S. vortens* cultivated in TYI-S-33 medium are pyriform (100X).

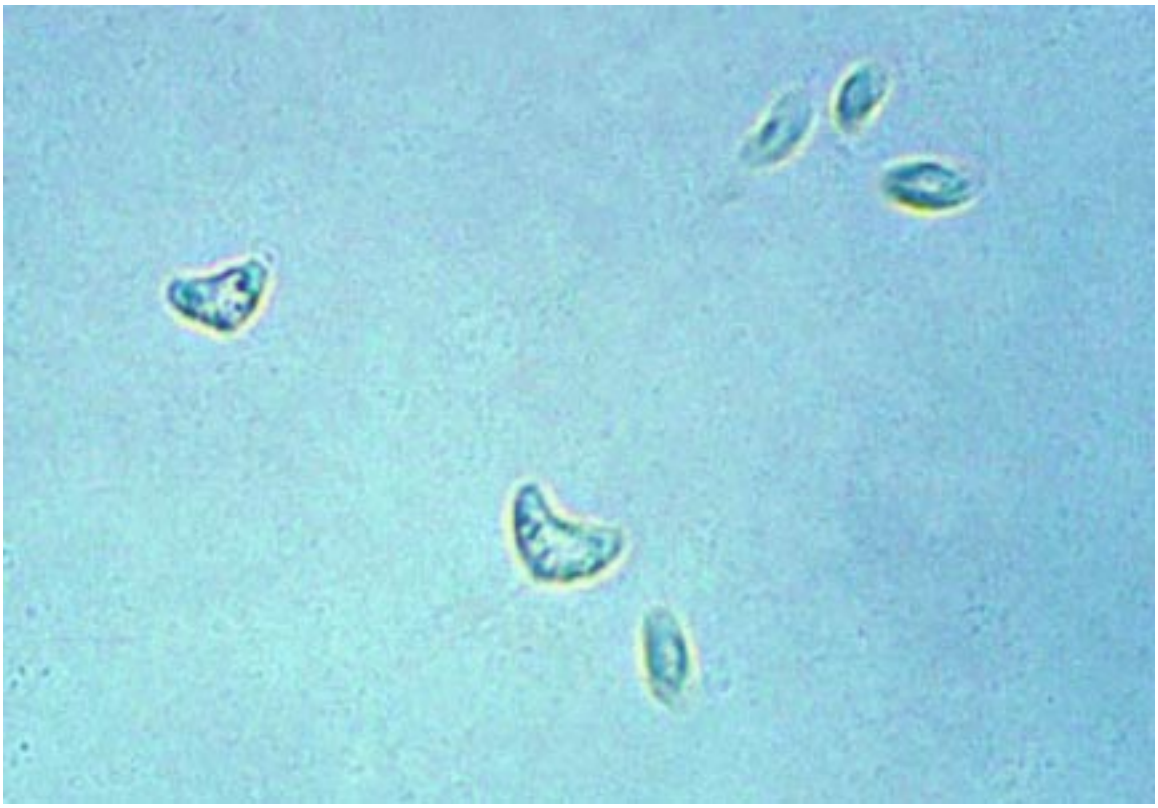

Figure 2-7. The v-shaped (dividing) trophozoites of *S. vortens* were found mostly during the log phase of growth (approximately on d2-d3 of cultivation) (40X).

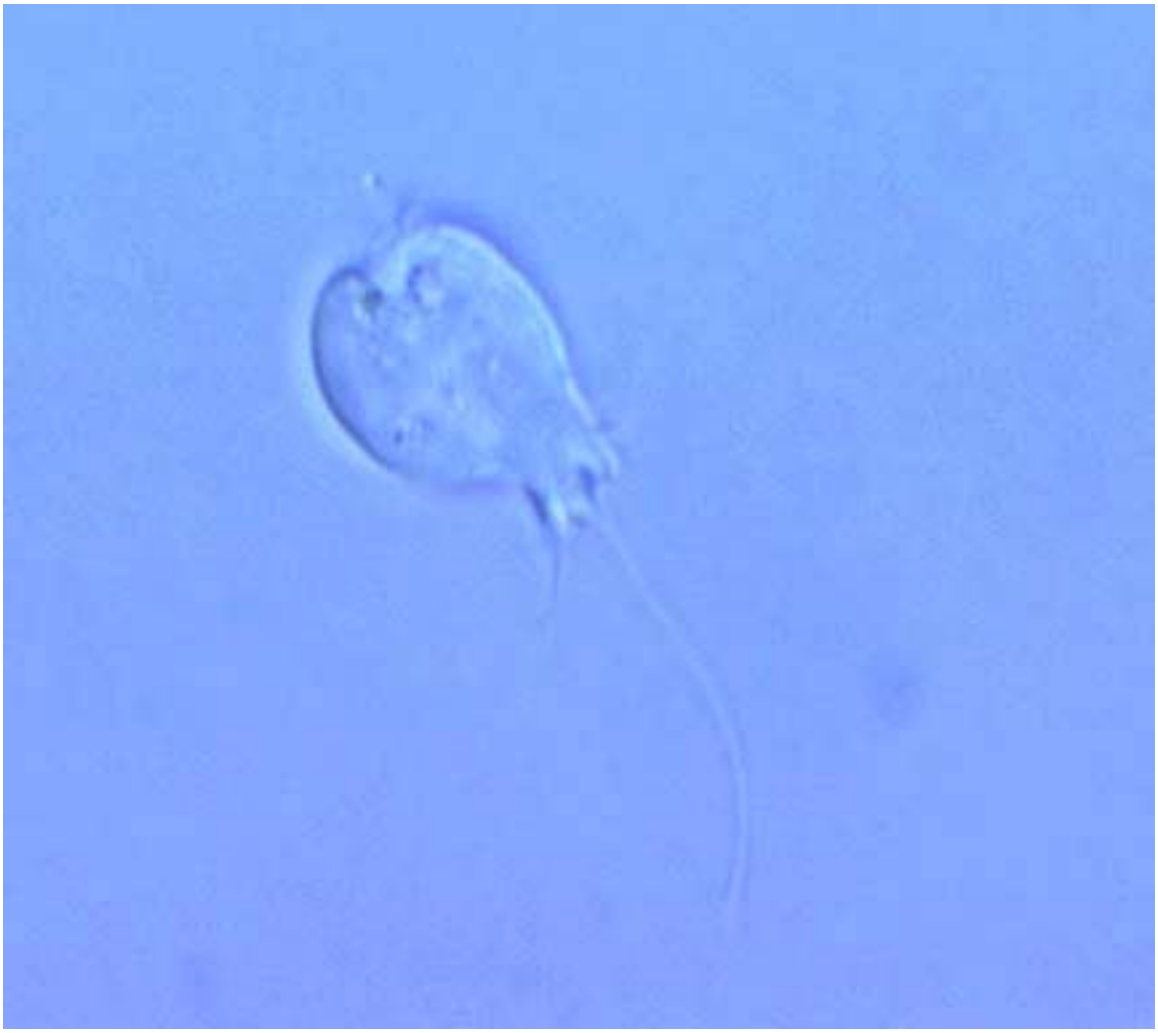

Figure 2-8. Another form of dividing trophozoite seen in the culture medium (100X).

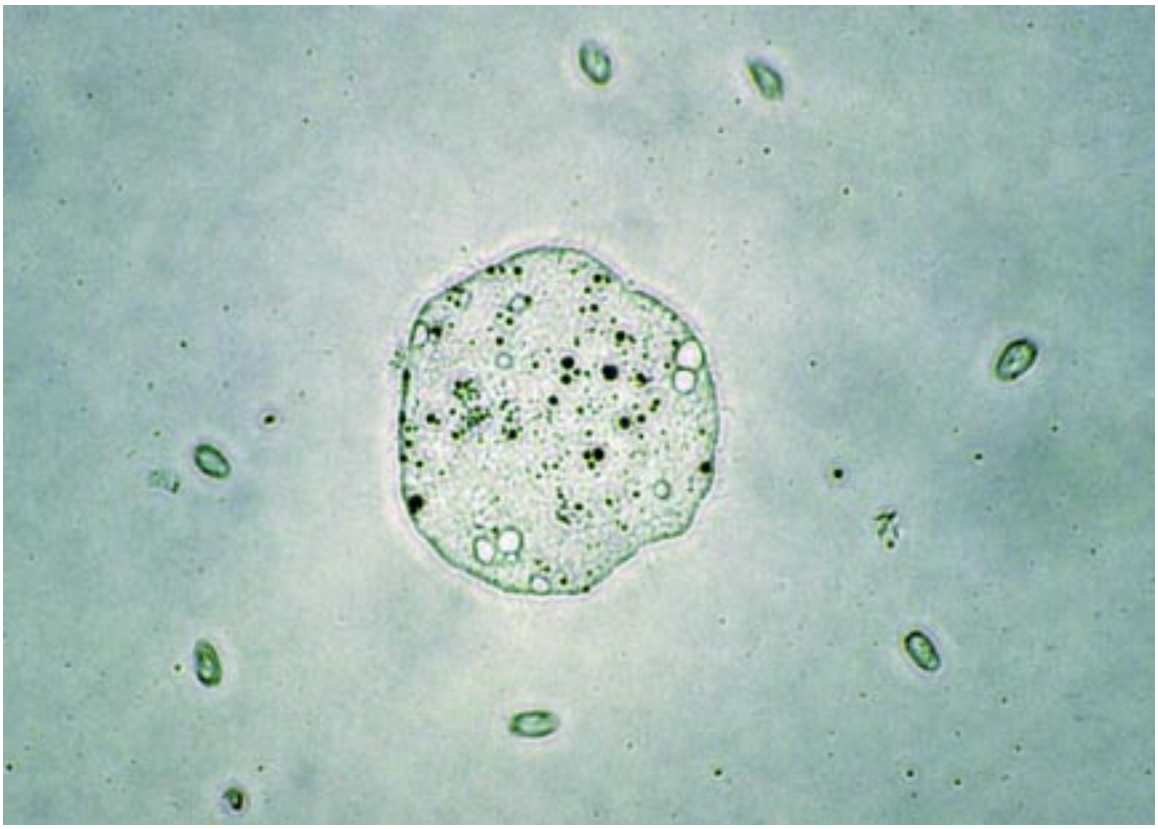

Figure 2-9. The mutinucleated (abnormal) trophozoite of *S. vortens* in culture medium (20X). This form was found only 1-2% in the routine culture.

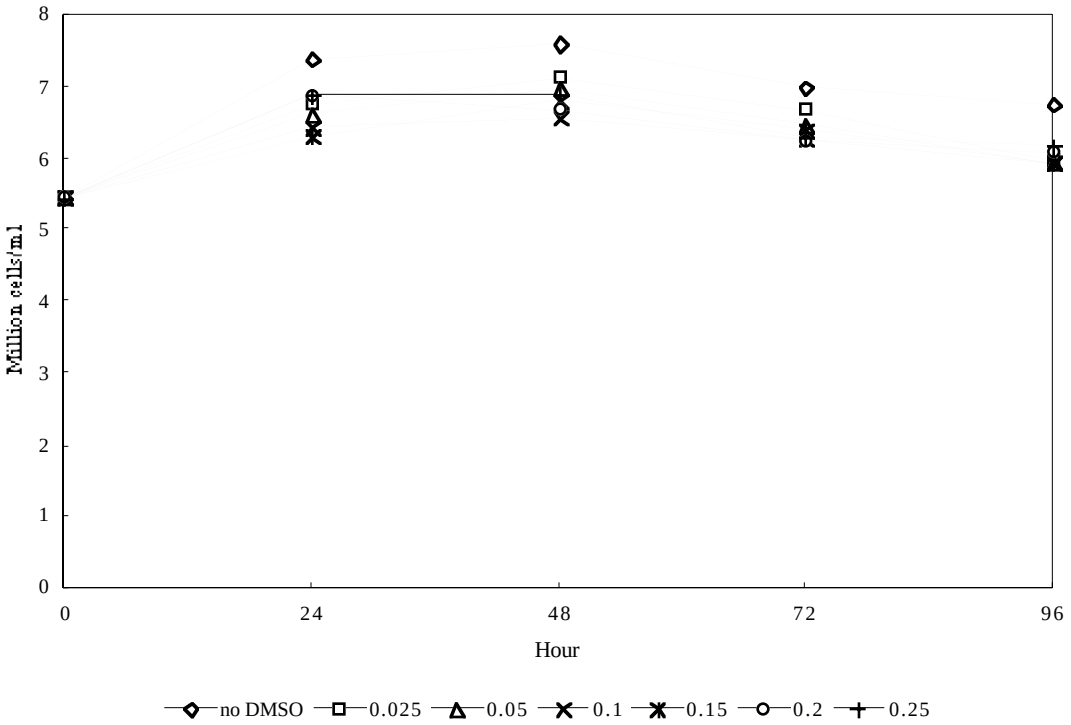

Figure 3-1. Growth of *S. vortens* cultivated in different concentrations (%) of DMSO (each value represents the mean of triplicate samples)

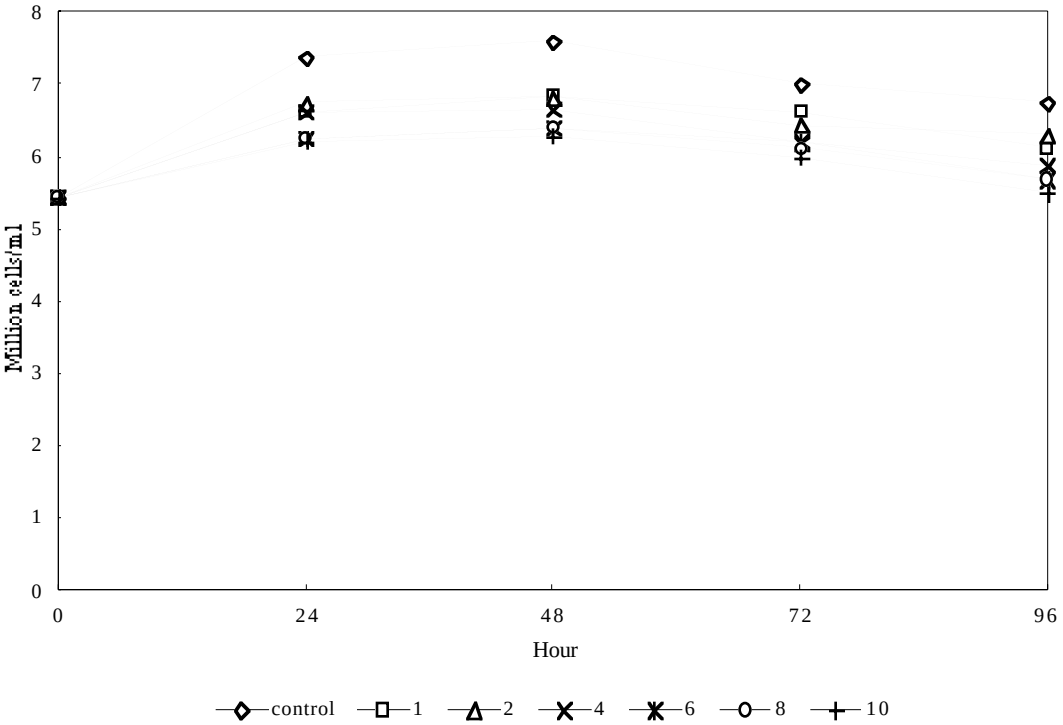

Figure 3-2. Growth of *S. vortens* cultivated in different concentrations (μg ml^{-1}) of pyrimethamine (each value represents the mean of triplicate samples)

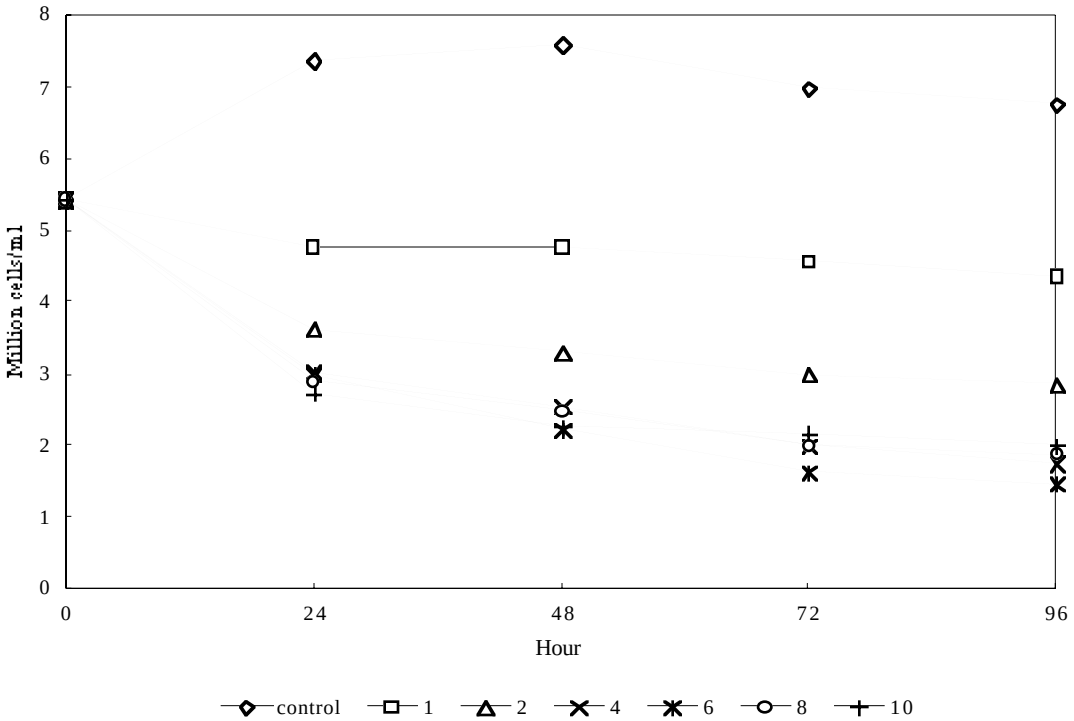

Figure 3-3. Growth of *S. vortens* cultivated in different concentrations (µg ml^{-1}) of dimetridazole (each value represents the mean of triplicate samples)

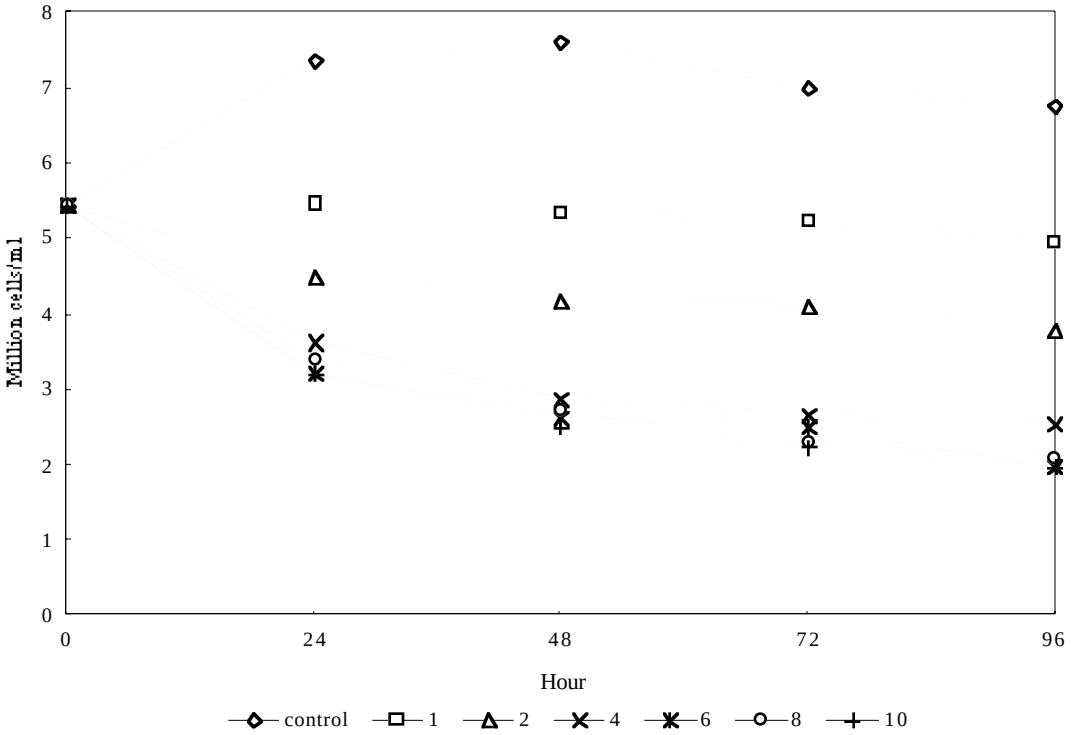

Figure 3-4. Growth of *S. vortens* cultivated in different concentrations ($\mu g\ ml^{-1}$) of metronidazole (each value represents the mean of triplicate samples)

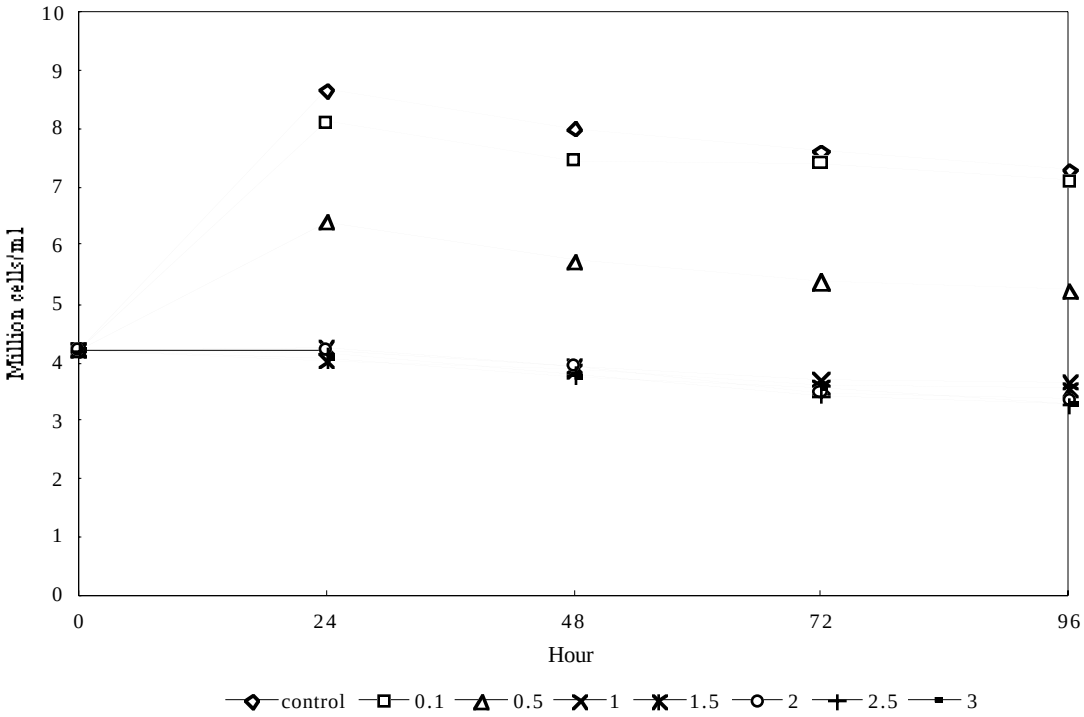

Figure 3-5. Growth of *S. vortens* cultivated in different concentrations (µg ml^{-1}) of albendazole (each value represents the mean of triplicate samples)

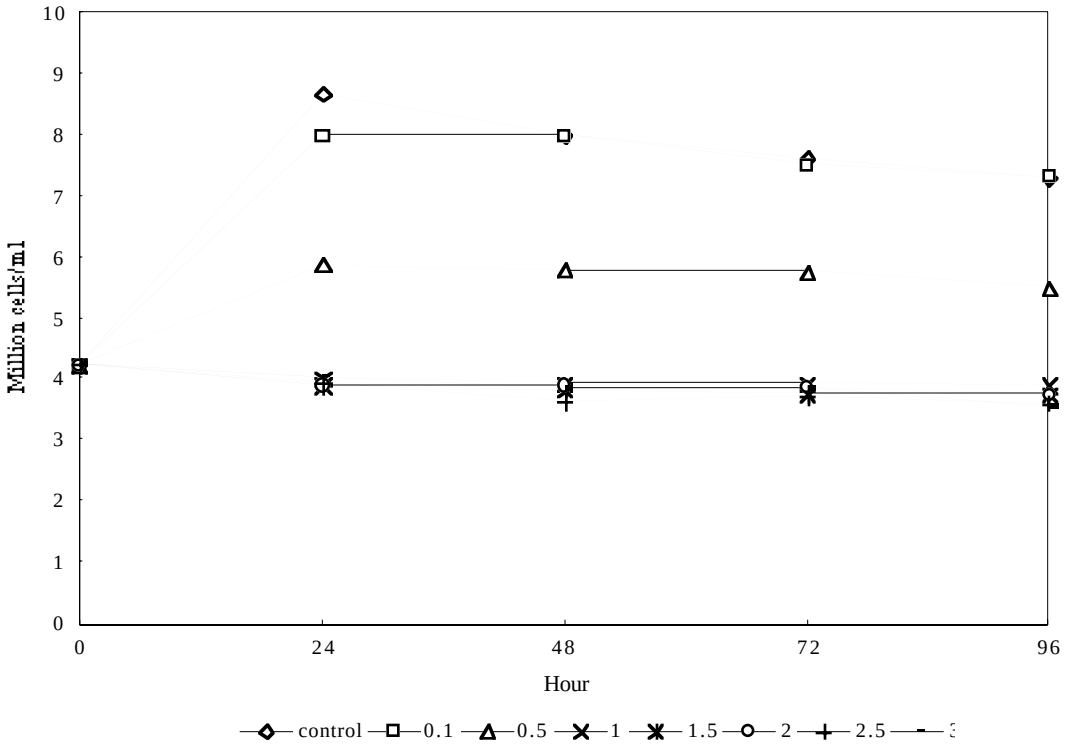

Figure 3-6. Growth of *S. vortens* cultivated in different concentrations (μg ml^{-1}) of fenbendazole (each value represents the mean of triplicate samples)

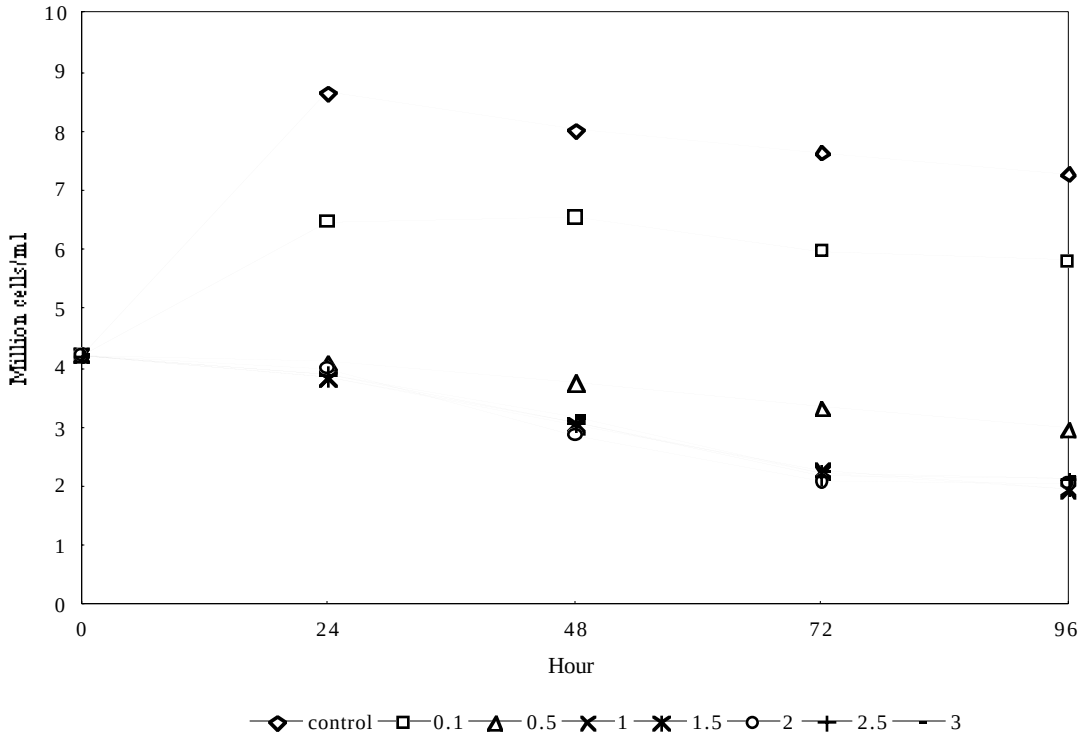

Figure 3-7. Growth of *S. vortens* cultivated in different concentrations (µg ml^{-1}) of mebendazole (each value represents the mean of triplicate samples)

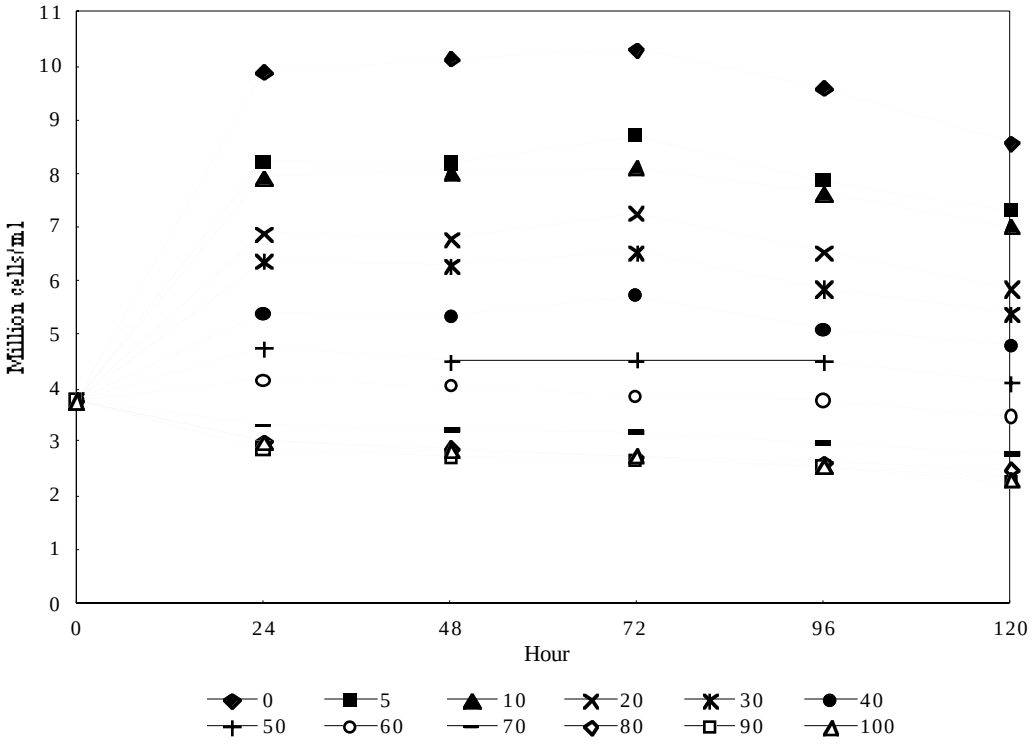

Figure 3-8. Growth of *S. vortens* cultivated in different concentrations (mg ml^{-1}) of $MgSO_4$ (each value represents the mean of triplicate samples)

Figure 4-1. Angelfish (*Pterophyllum scalare*) raised in the Aquatic Medicine Laboratory of the VMRCVM and used in the research.

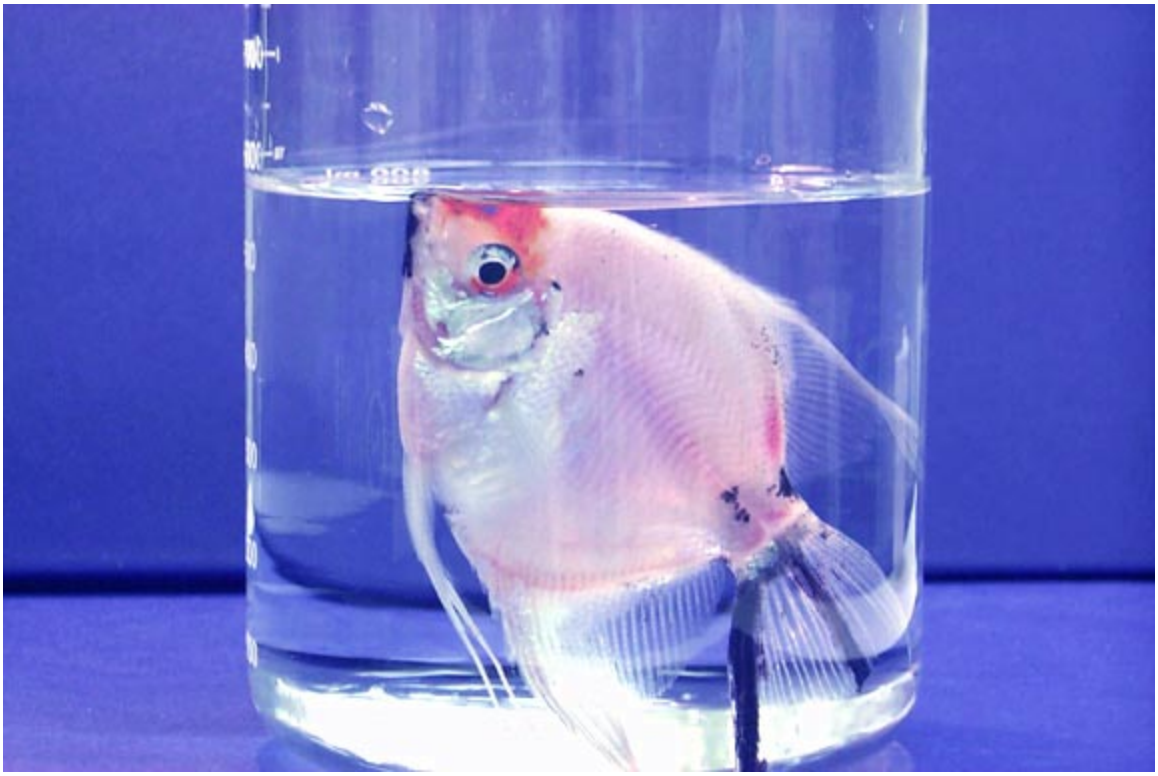

Figure 4-2. An angelfish undergoing anaesthesia with tricane methanesulfonate (MS-222).

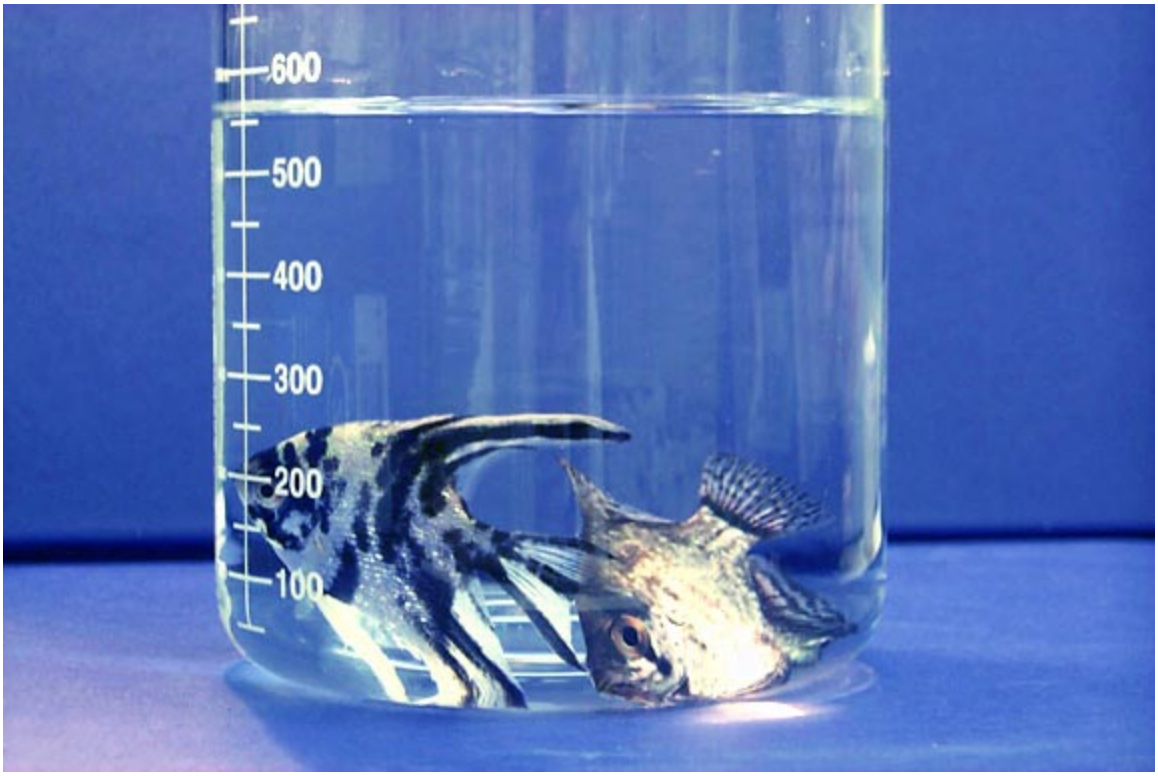

Figure 4-3. Mildly anaesthetized angelfish which were then orally or intraperitoneally inoculated with *S. vortens*.

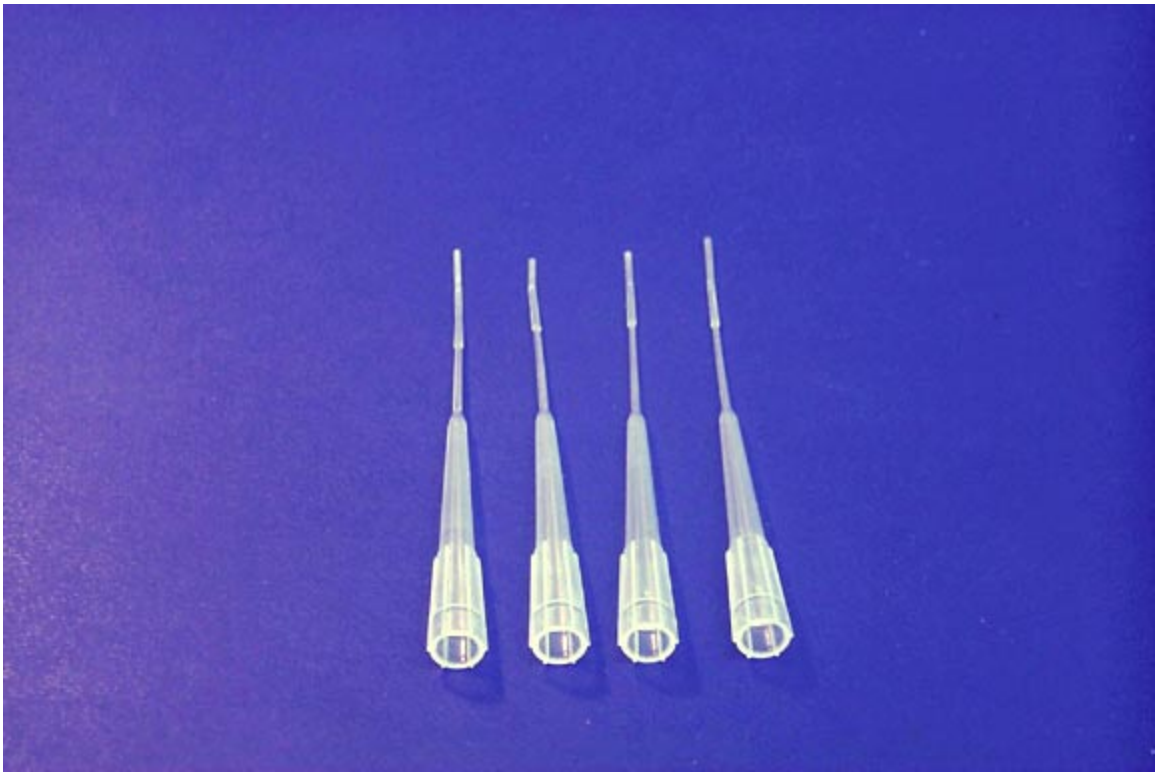

Figure 4-4. Micropipette tips attached with small plastic tubes were used for oral inoculation of *S. vortens* into angelfish.

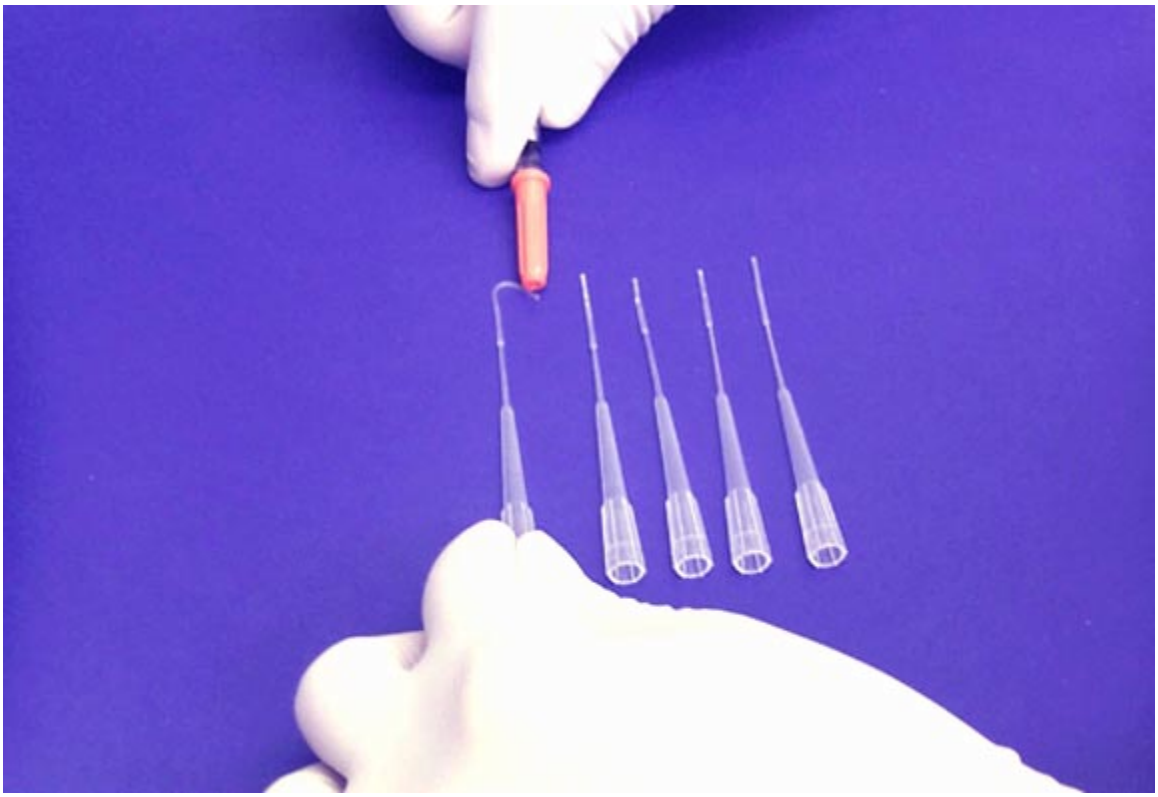

Figure 4-5. The flexible soft-tube attached on the micropipette tip prevented damage to the angelfish's esophagus during oral inoculation of *S. vortens*.

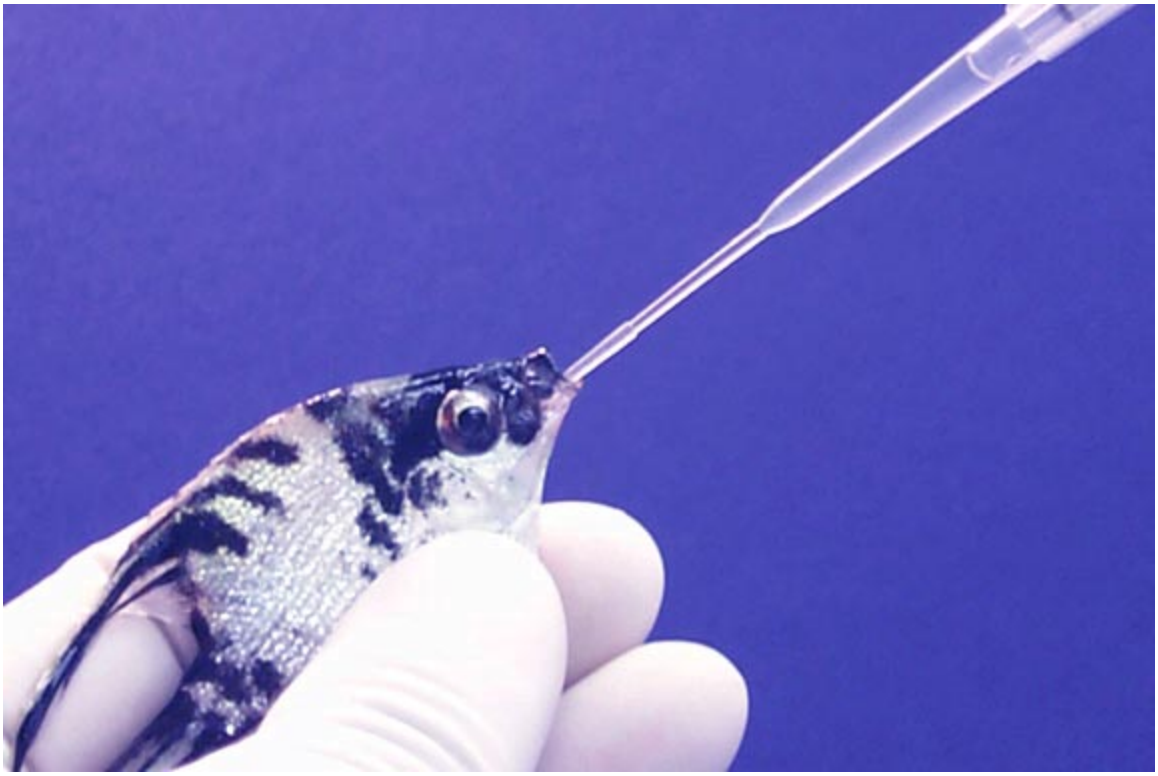

Figure 4-6. Oral inoculation of *S. vortens* into an anesthetized angelfish.

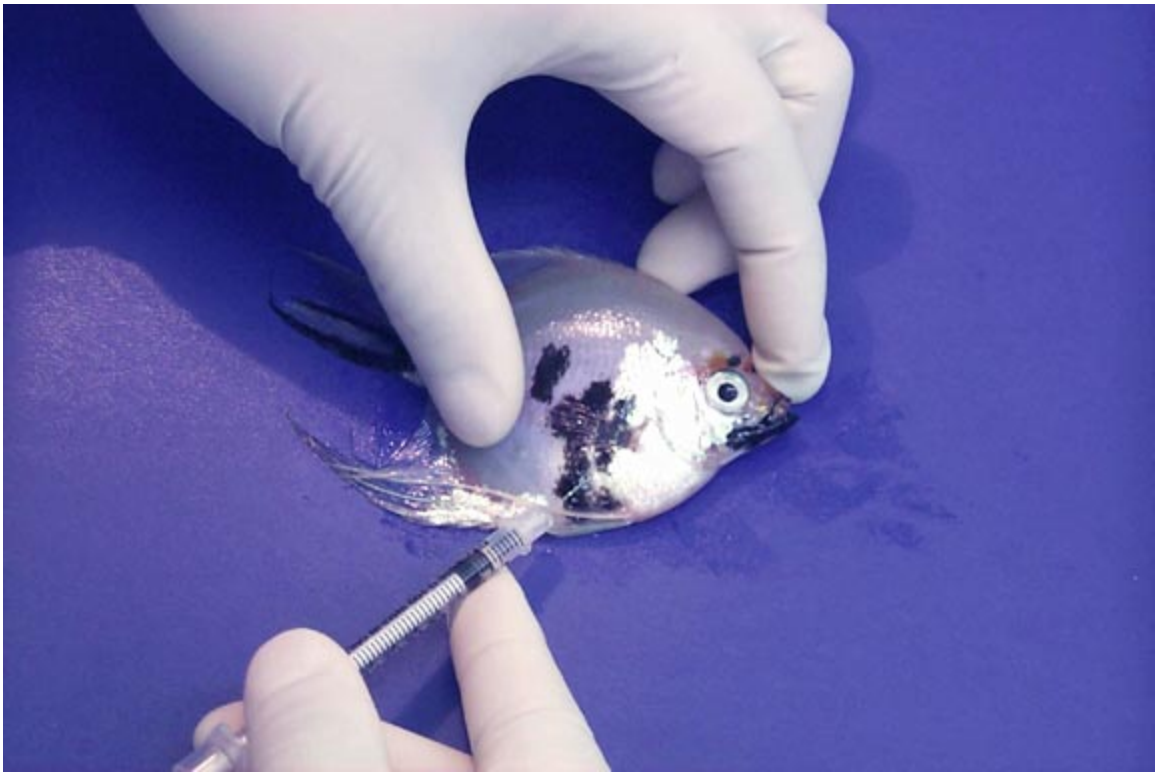

Figure 4-7. Intraperitoneal inoculation of *S. vortens* into an angelfish.

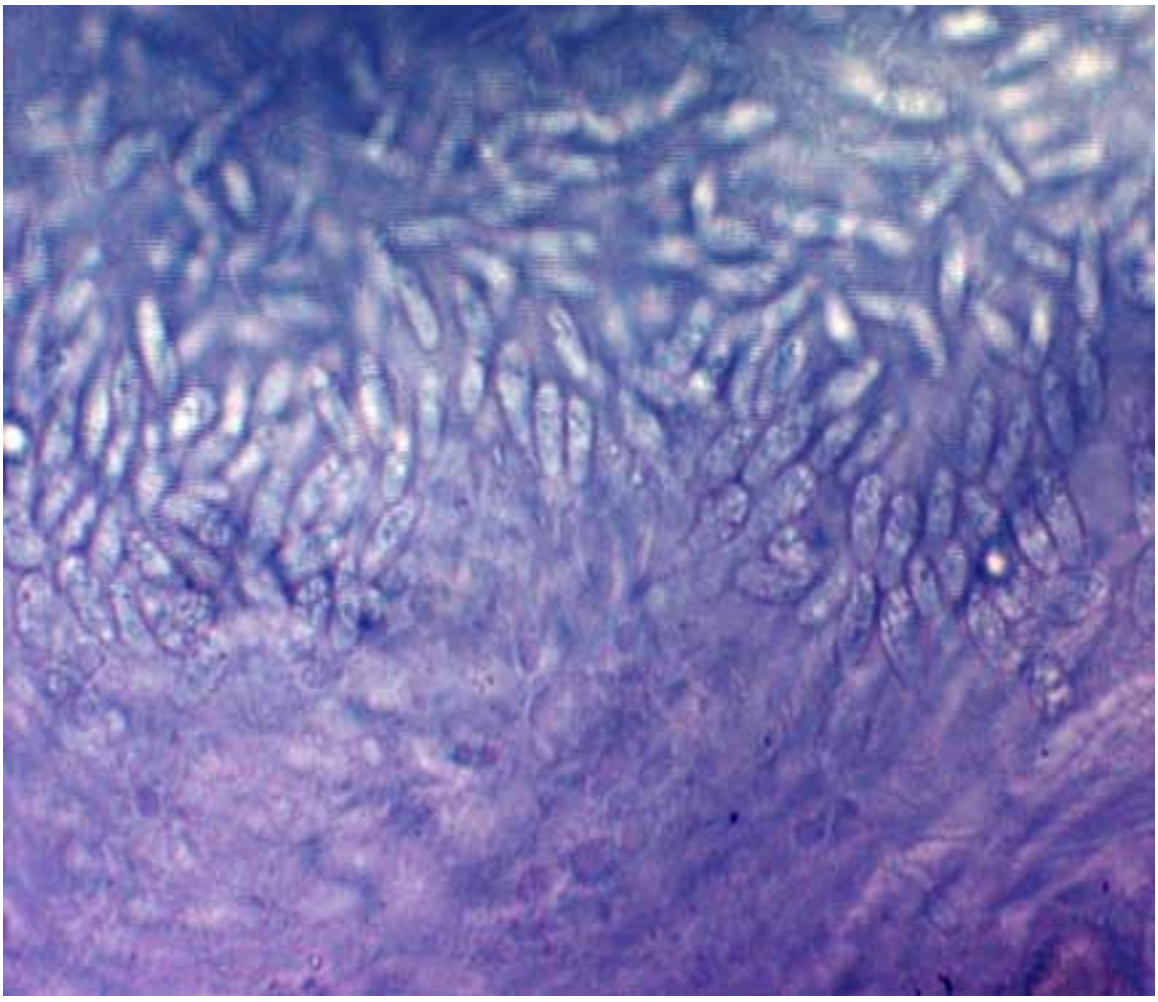

Figure 4-8. Large number of *S. vortens* were found in the middle to posterior intestine of the angelfish. Fresh impression smear of angelfish intestine (20X).

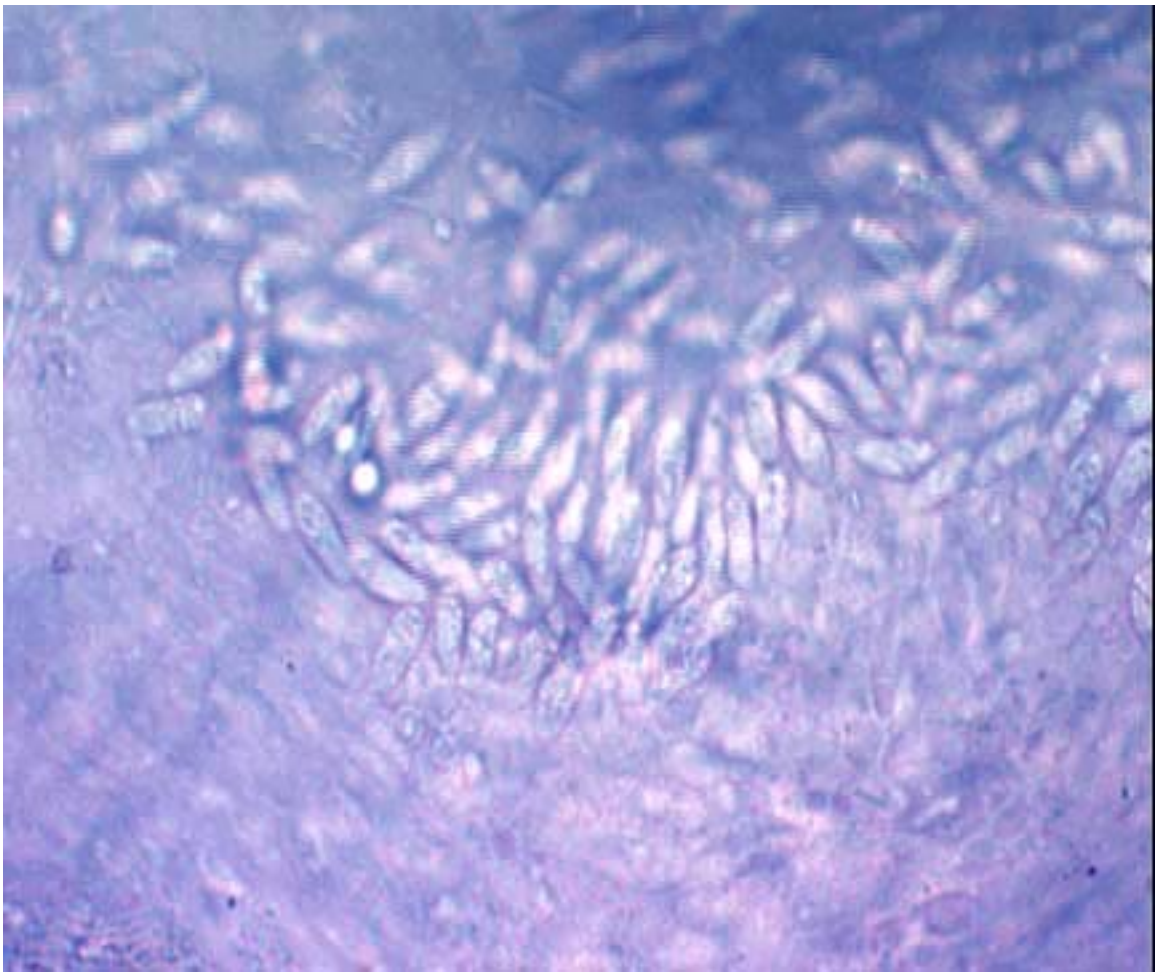

Figure 4-9. Aggregation of *S. vortens* on the intestinal mucosa. Fresh impression smear of angelfish intestine (40X).

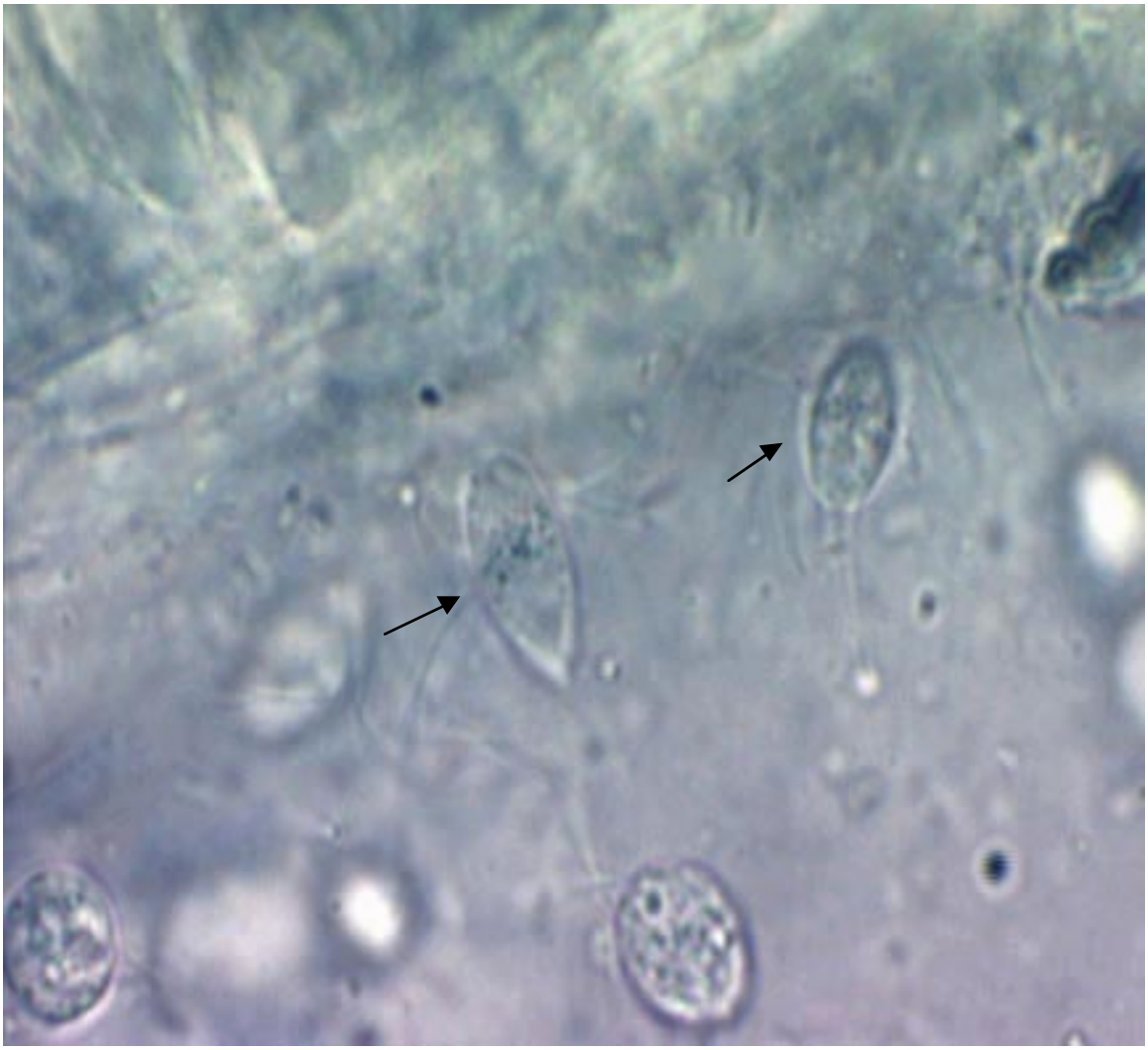

Figure 4-10. *Spironucleus vortens* (arrows) appeared attached to the intestinal mucosa. Fresh impression smear of angelfish intestine (100X).

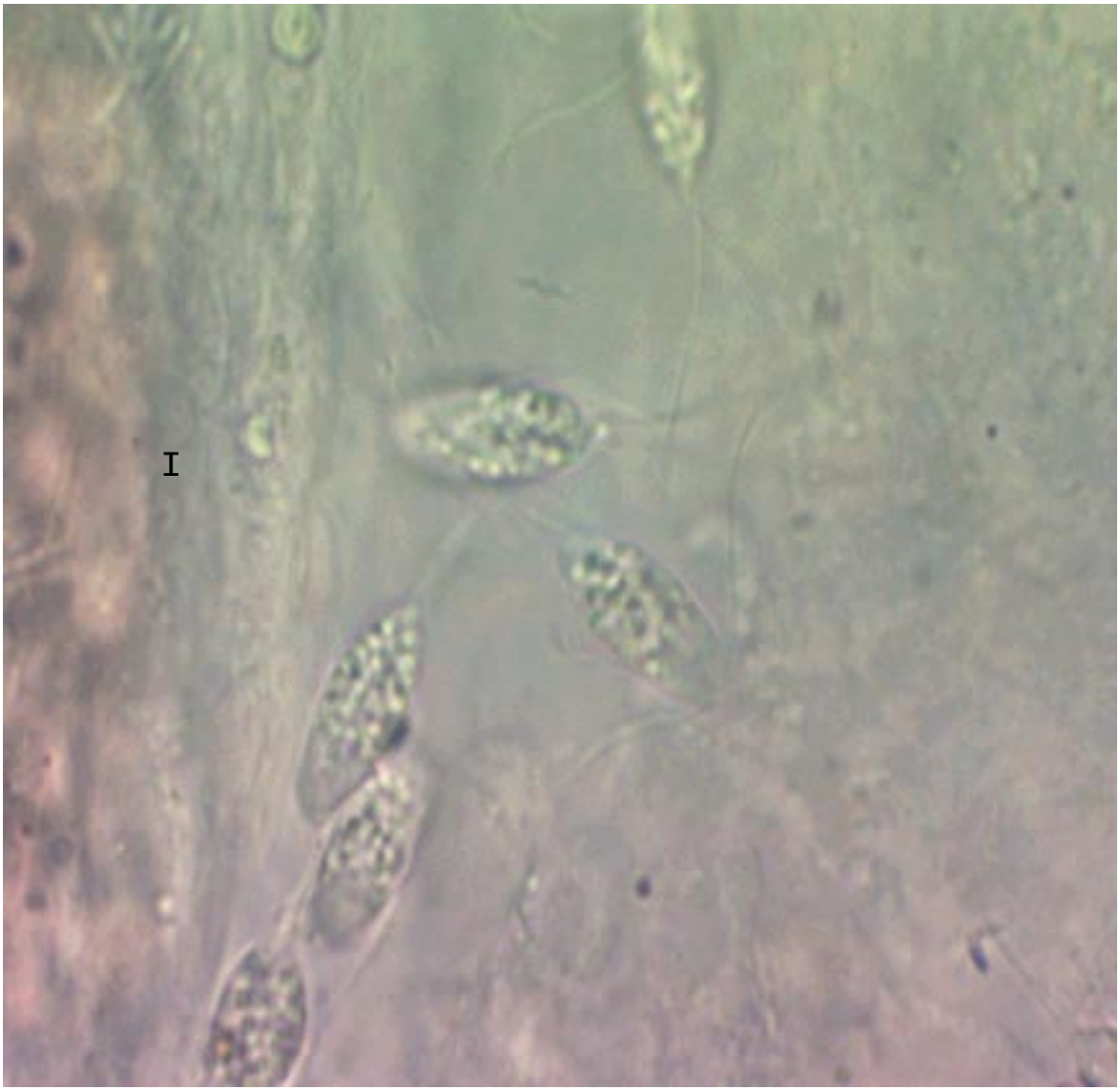

Figure 4-11. The majority of *S. vortens* moved toward (histotrophic) the intestinal mucosa (I). Non-staining structures (granules) were observed inside the cells. Fresh impression smear of angelfish intestine (100X).

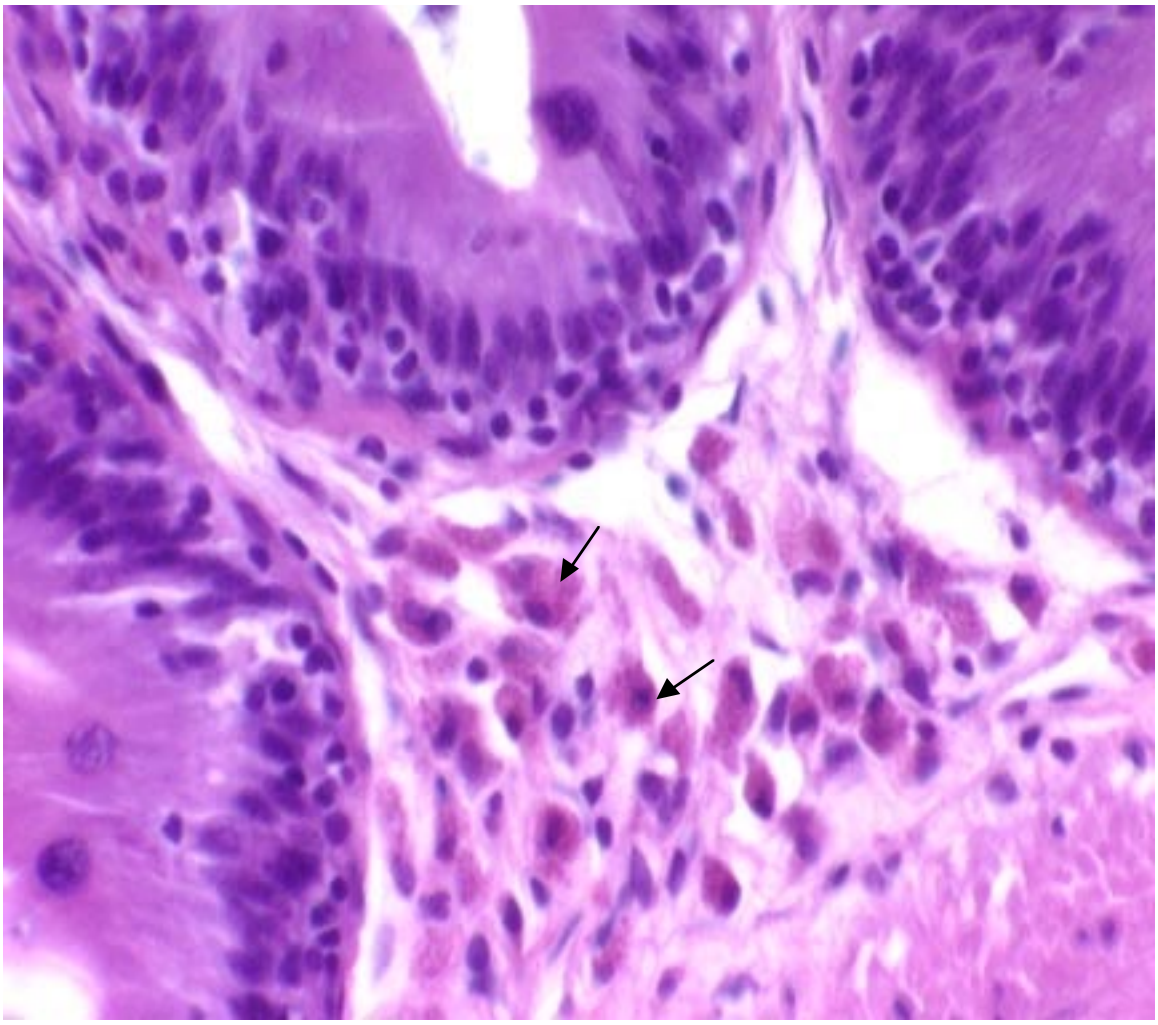

Figure 4-12. Eosinophilic granulocytes (arrows) were frequently observed within the intestinal submucosa of *S. vortens*-infected angelfish (H&E, 40X).

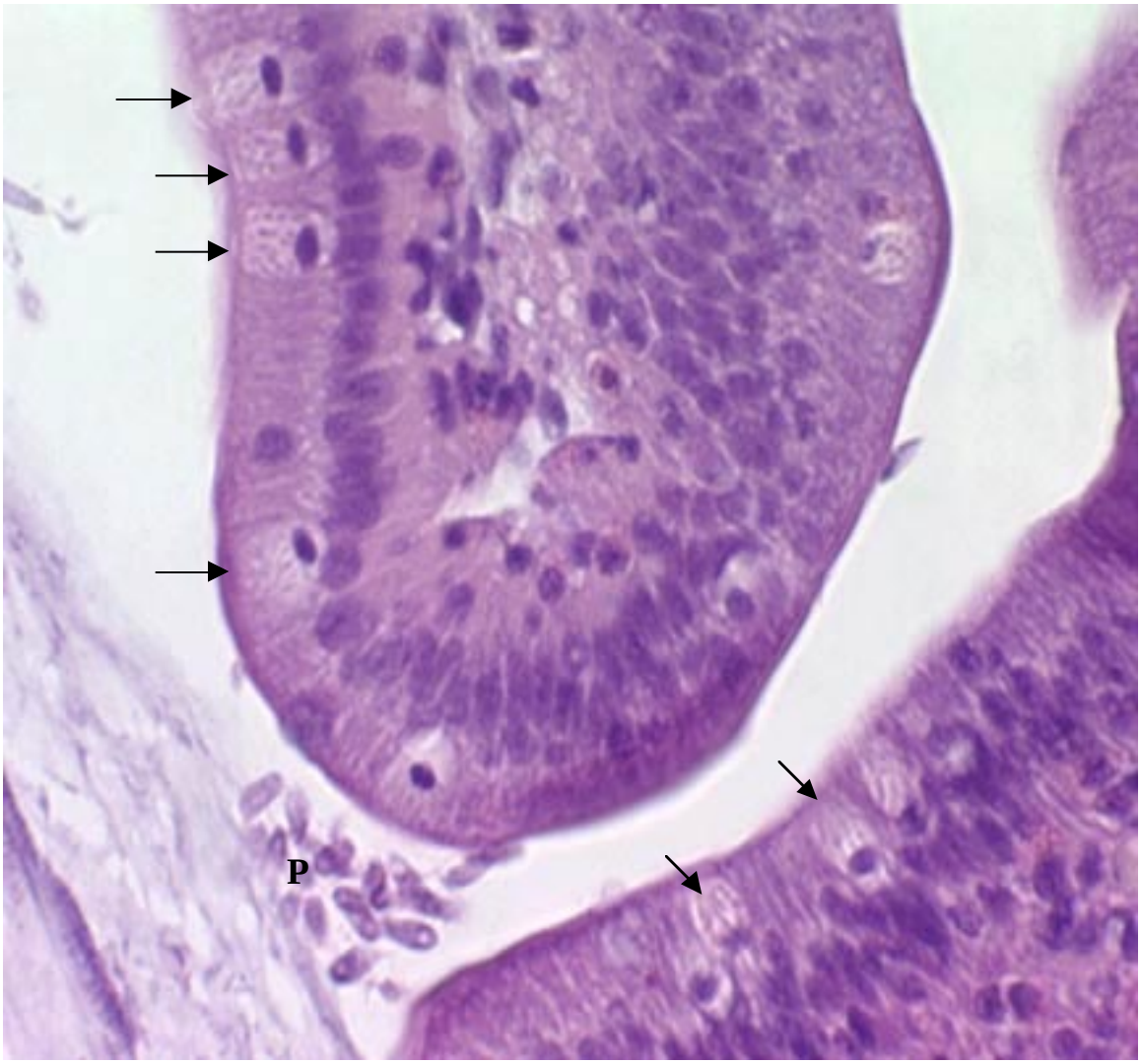

Figure 4-13. Many rodlet cells (arrows) were observed in the intestinal mucosa of infected angelfish. *Spironucleus vortens* (P) were located closely to the intestinal mucosa (H&E, 40X).

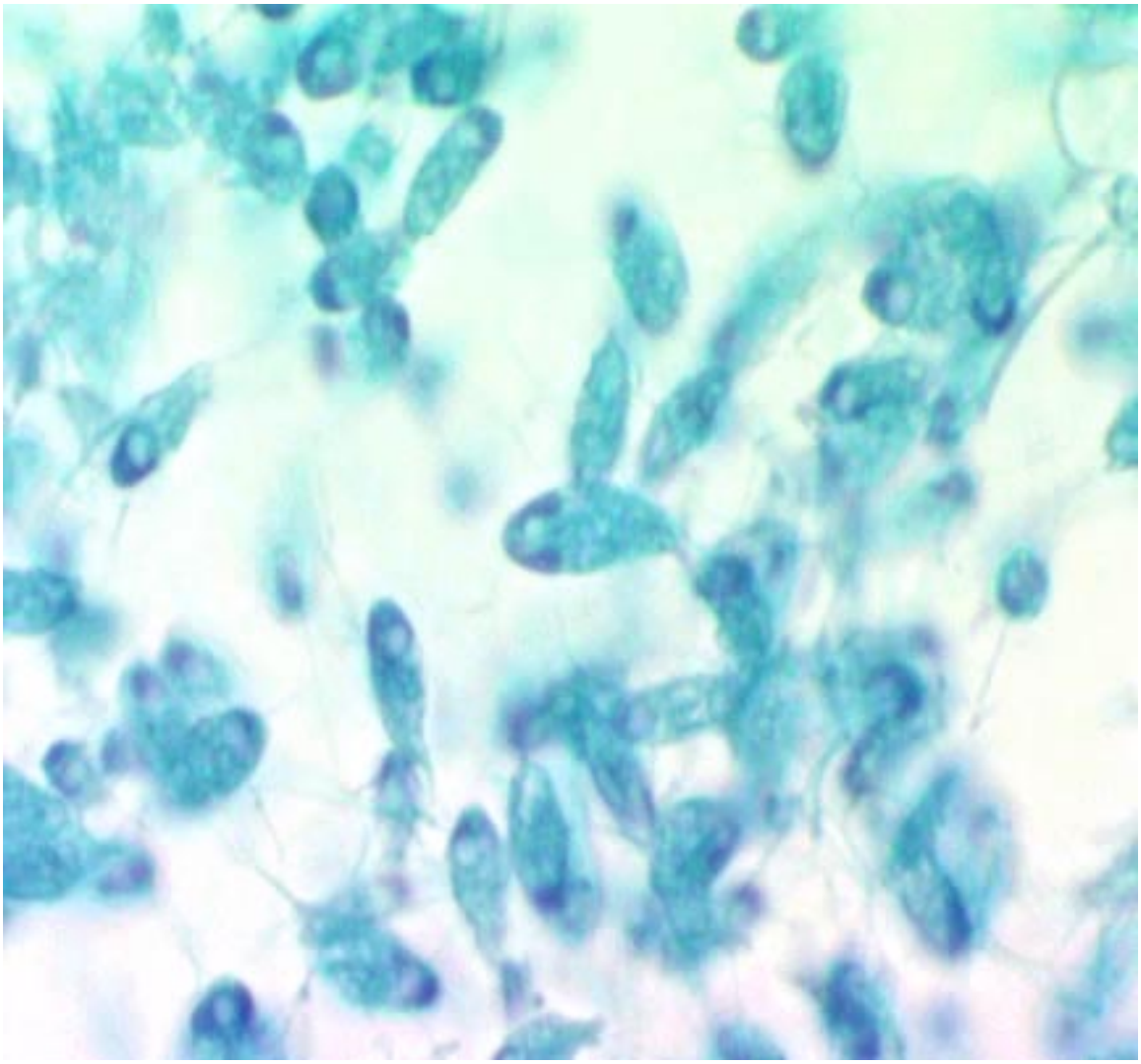

Figure 4-14. *Spironucleus vortens* in the intestinal lumen were pyriform in shape with two sausage-shaped nuclei located in the anterior one third of the cells (Feulgen staining, 100X).

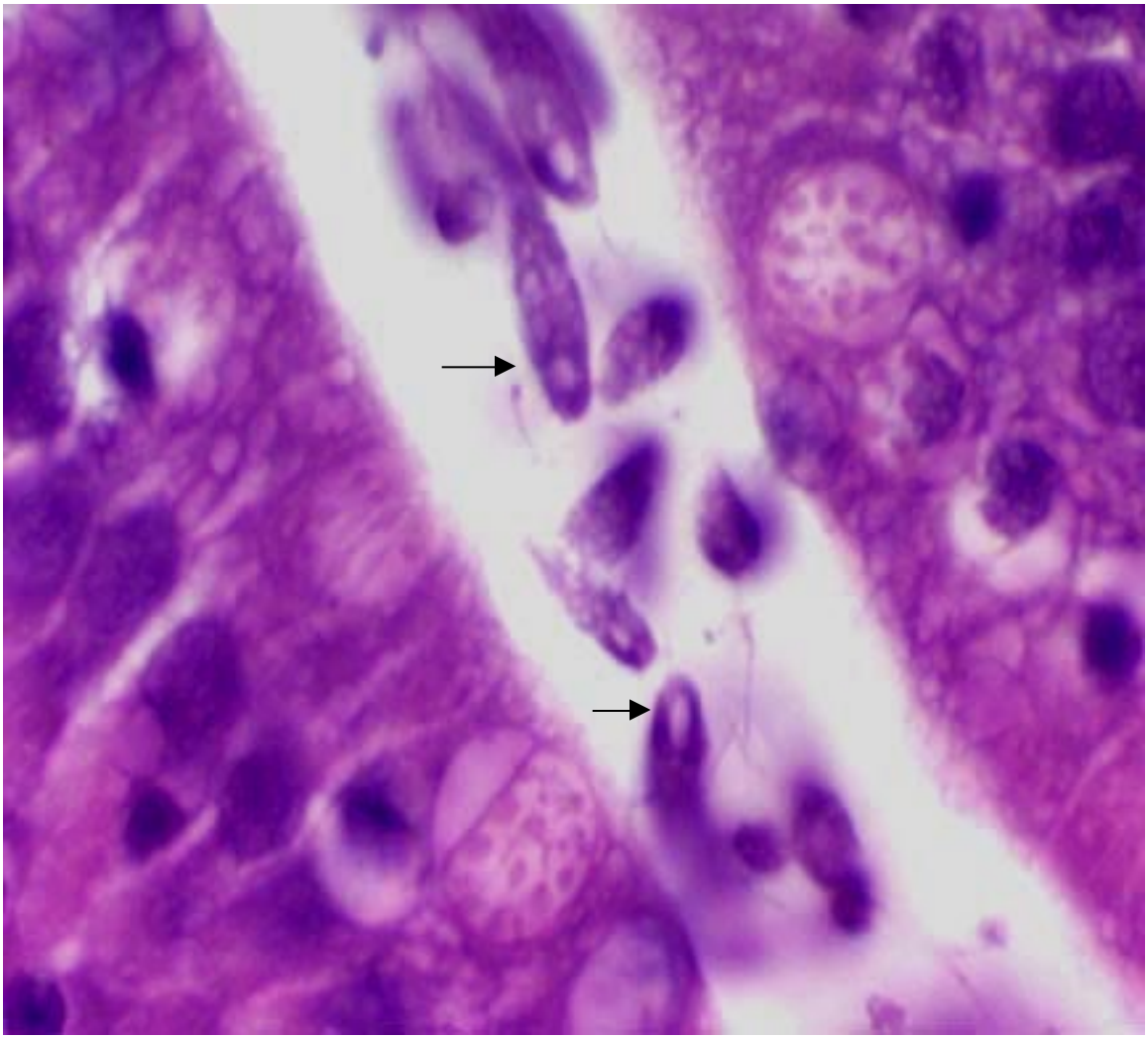

Figure 4-15. *Spironucleus vortens* (arrows) had anterior and posterior flagella, and non-staining cytoplasmic structures resembling vacuoles (H&E, 100X).

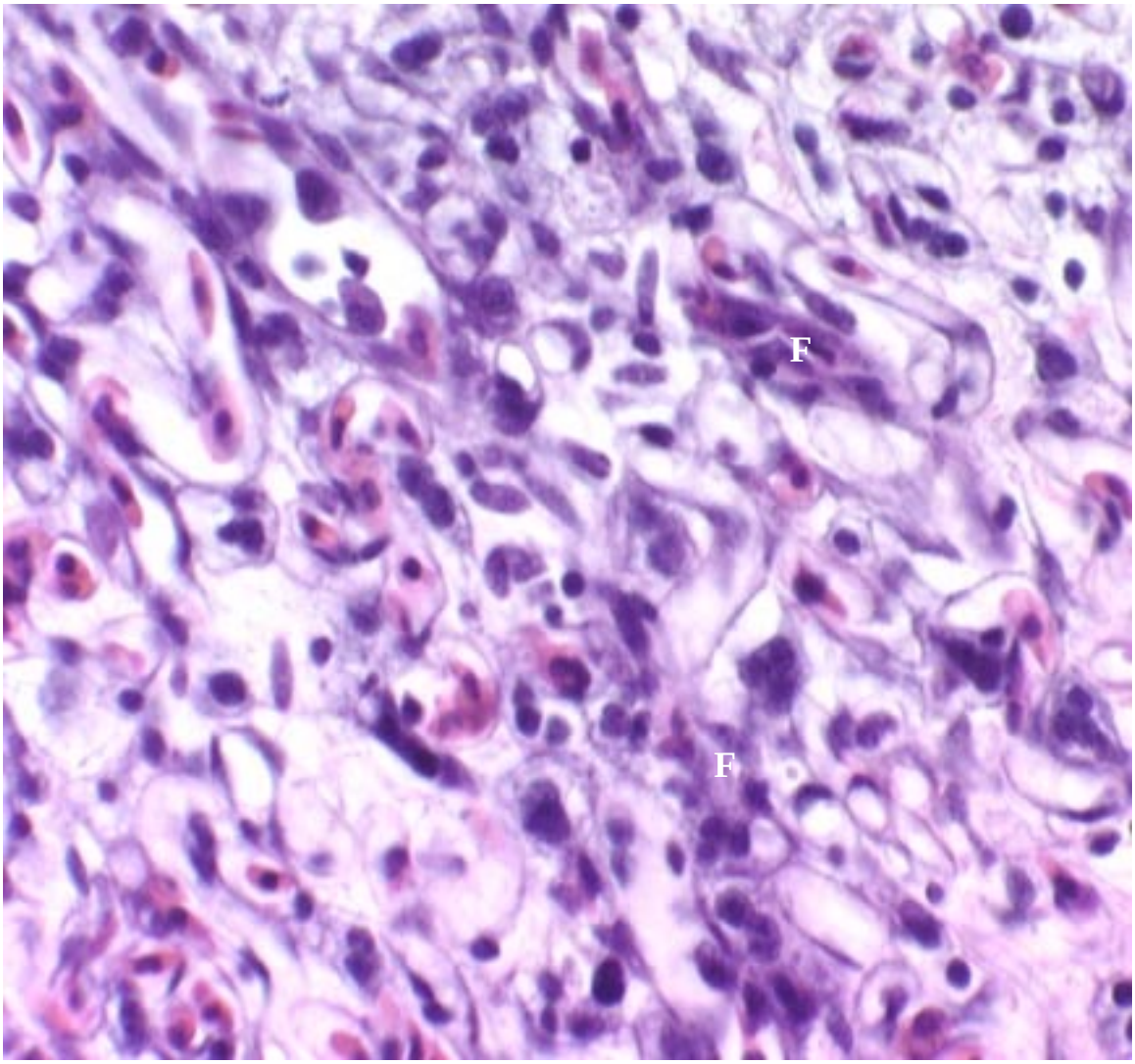

Figure 4-16. Fibroblast (F) proliferation within the mesentery (H&E, 40X).

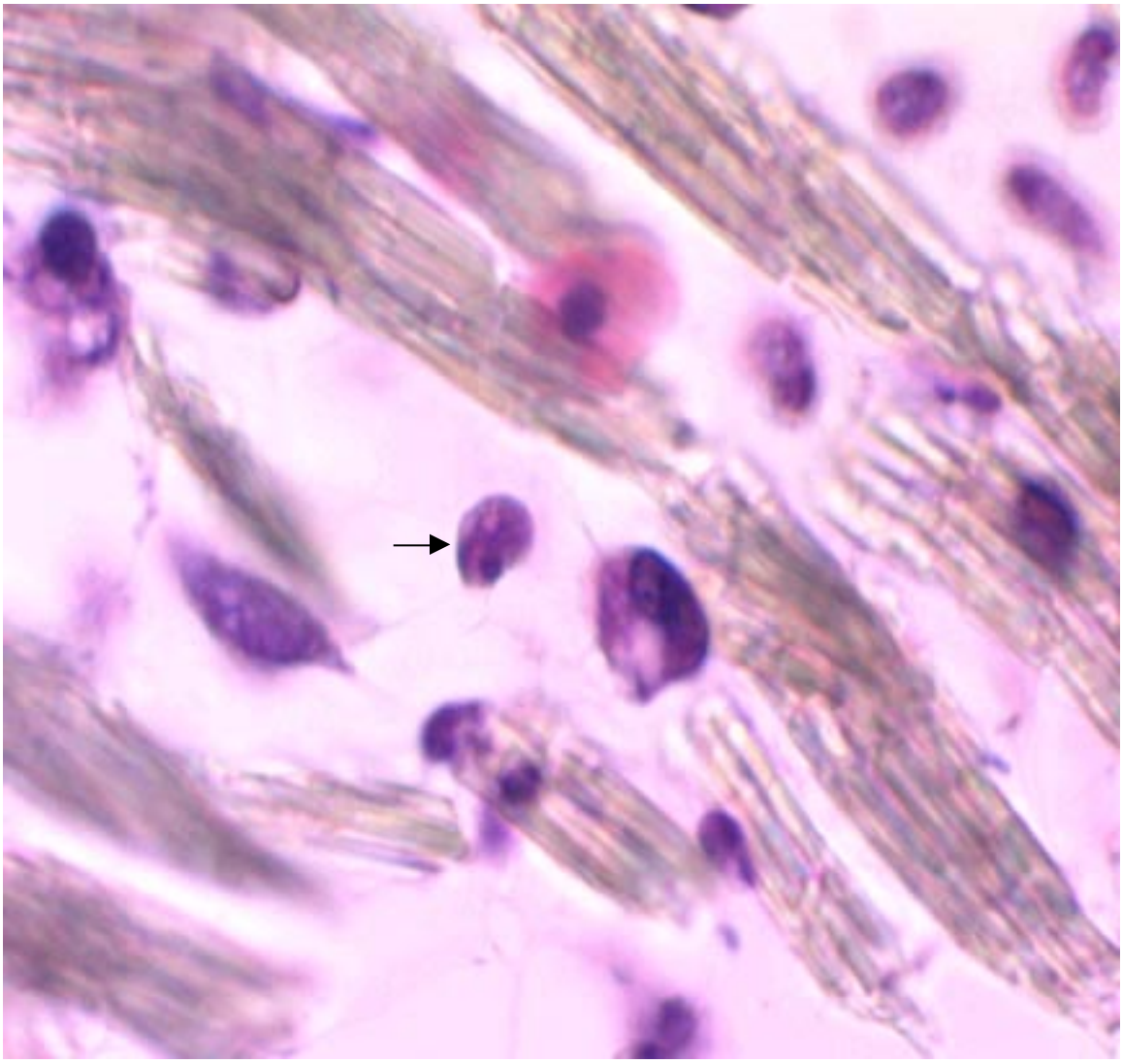

Figure 4-17. Round to oval-shaped *S. vortens* (arrow) were found within the abdominal cavity associated with inflammatory reaction (H&E, 100X).

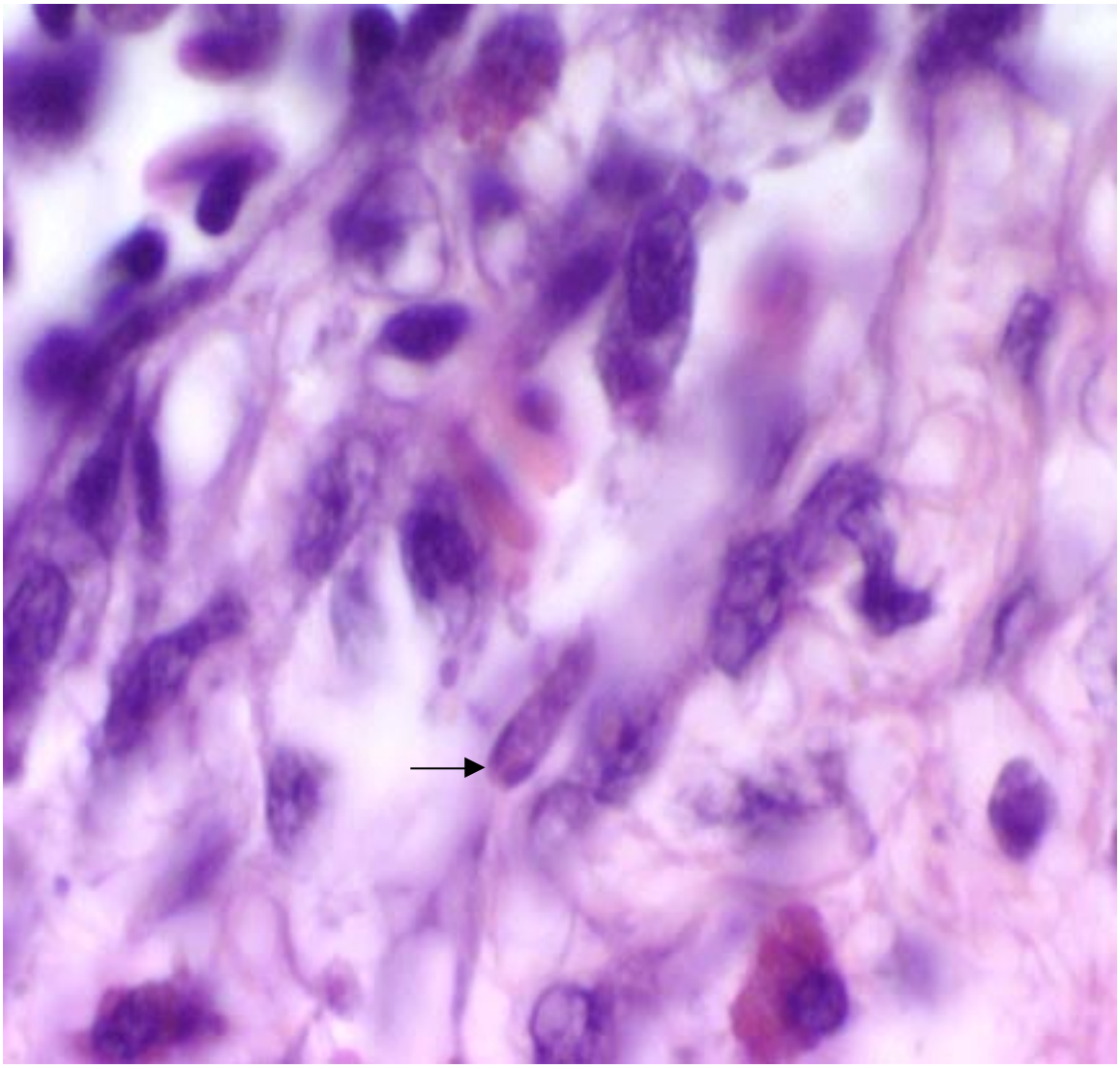

Figure 4-18. *Spironucleus vortens* (arrow) located in a mesenteric blood vessel (H&E, 100X).

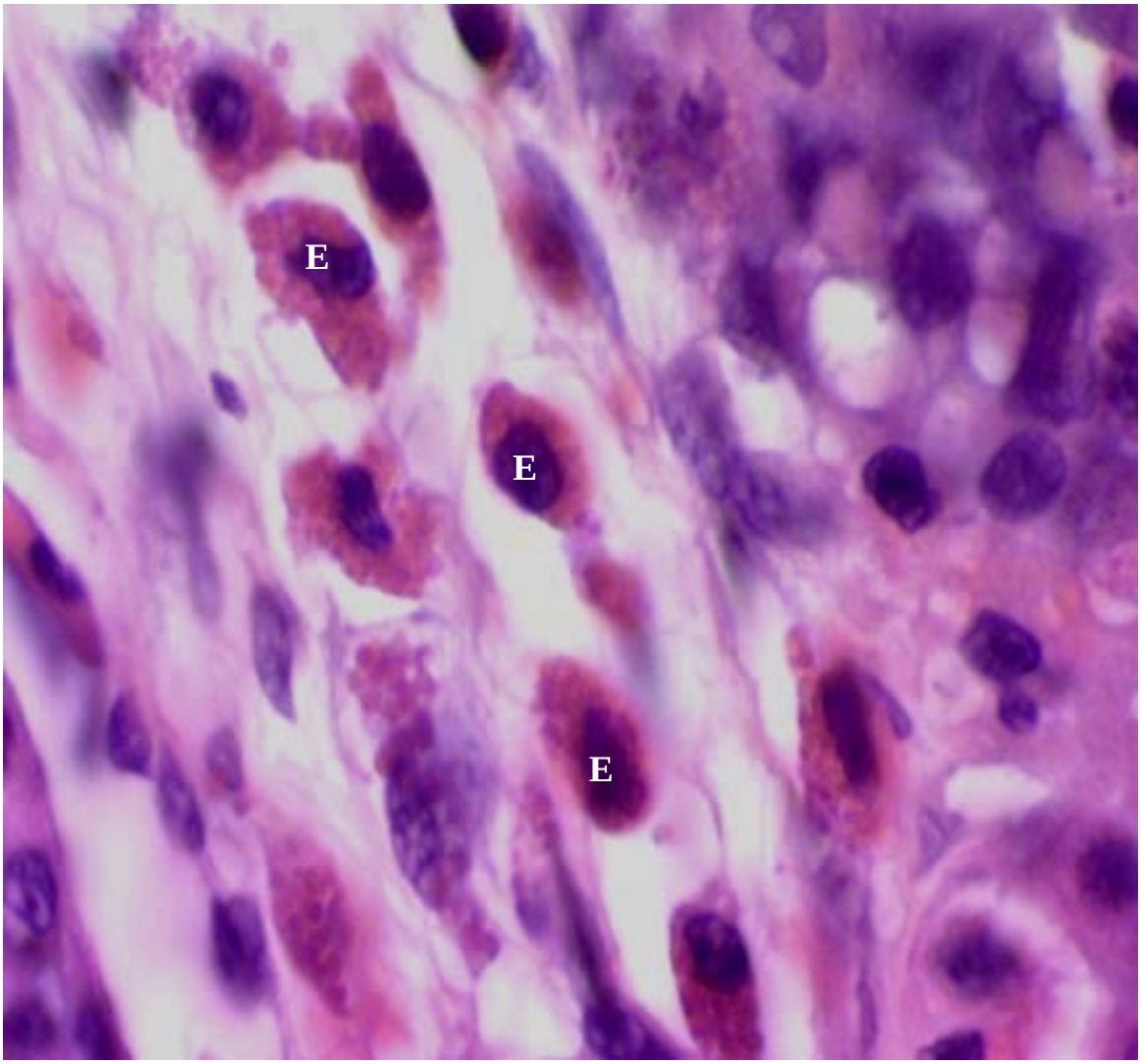

Figure 4-19. An increased number of eosinophilic granulocytes (E) were found in the intestinal submucosa of infected angelfish (H&E, 100X).

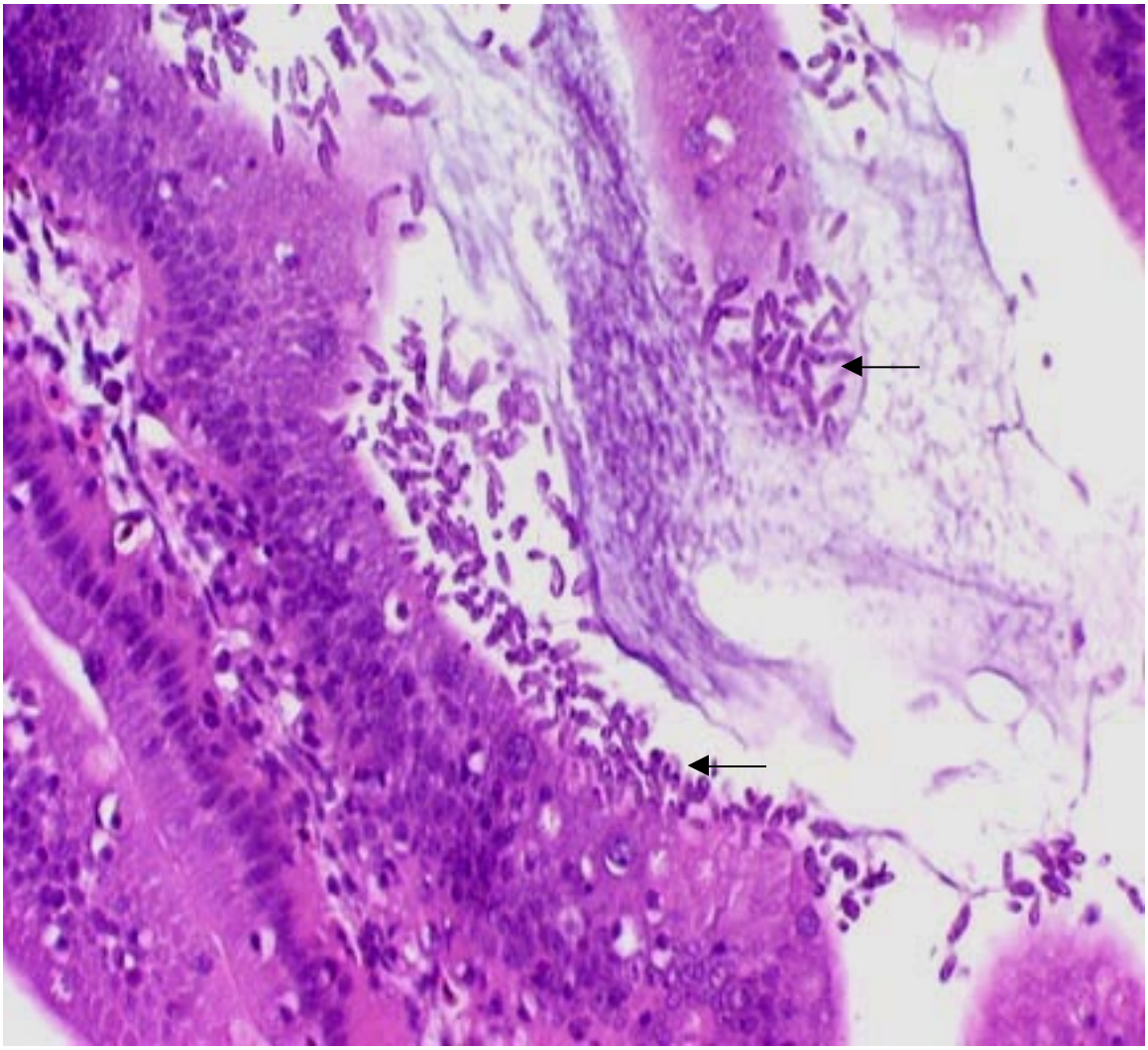

Figure 4-20. *Spironucleus vortens* (arrows) located in the intestinal lumen and along the intestinal epithelium (H&E, 20X).

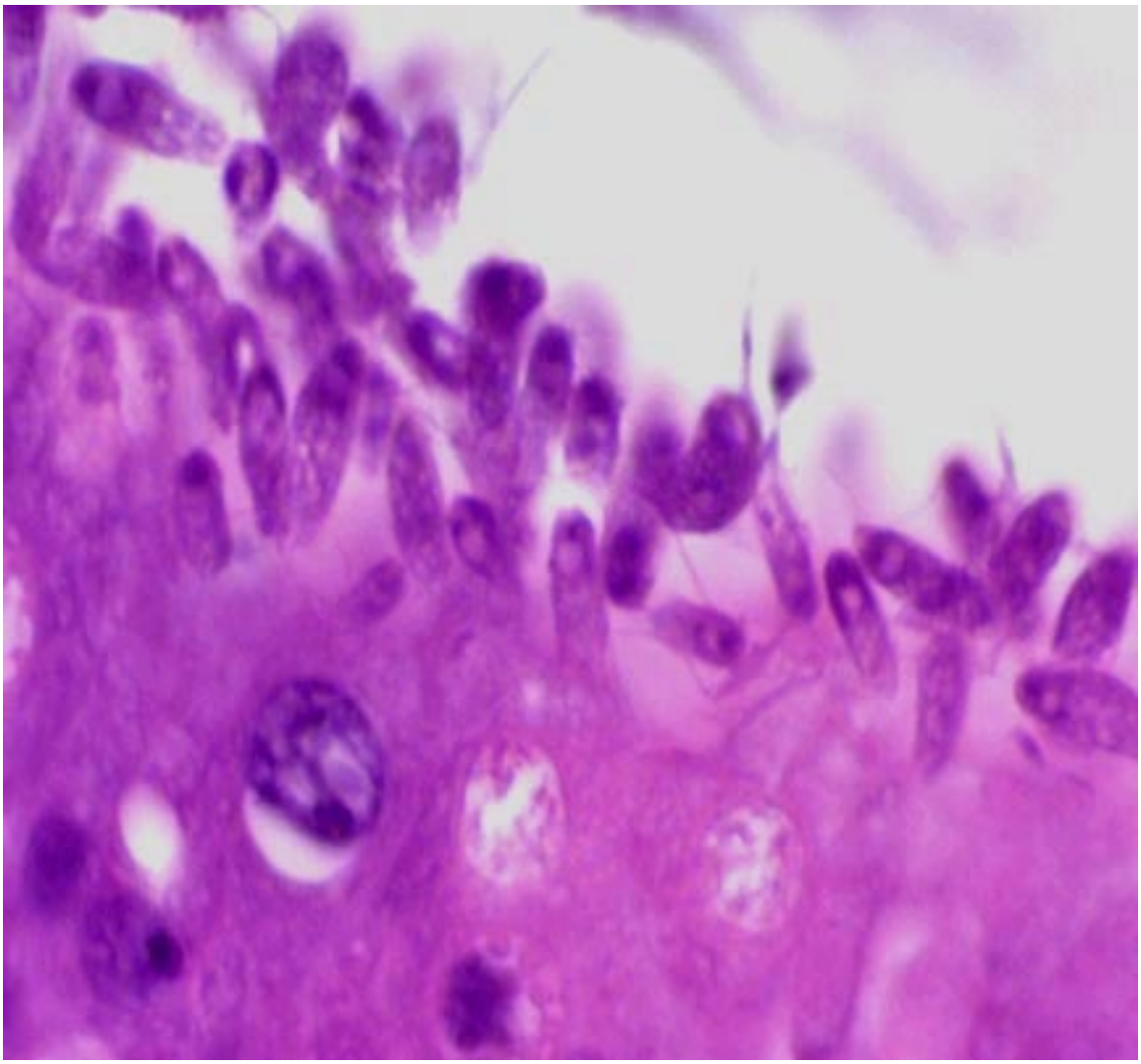

Figure 4-21. Most of the intra-luminal organisms were located adjacent to the intestinal mucosa (H&E, 100X).

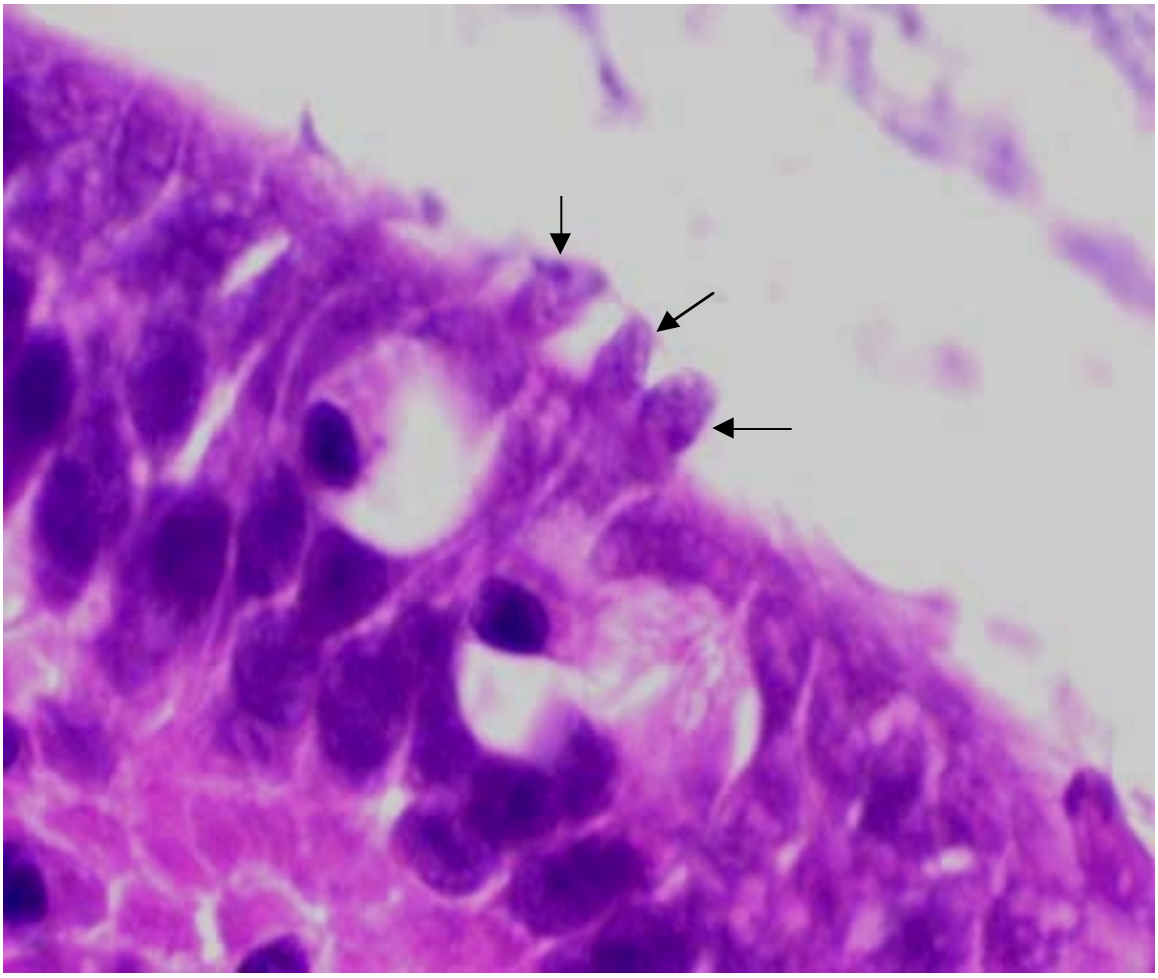

Figure 4-22. The *S. vortens* (arrows) appeared attached to the intestinal mucosa (H&E, 100X).

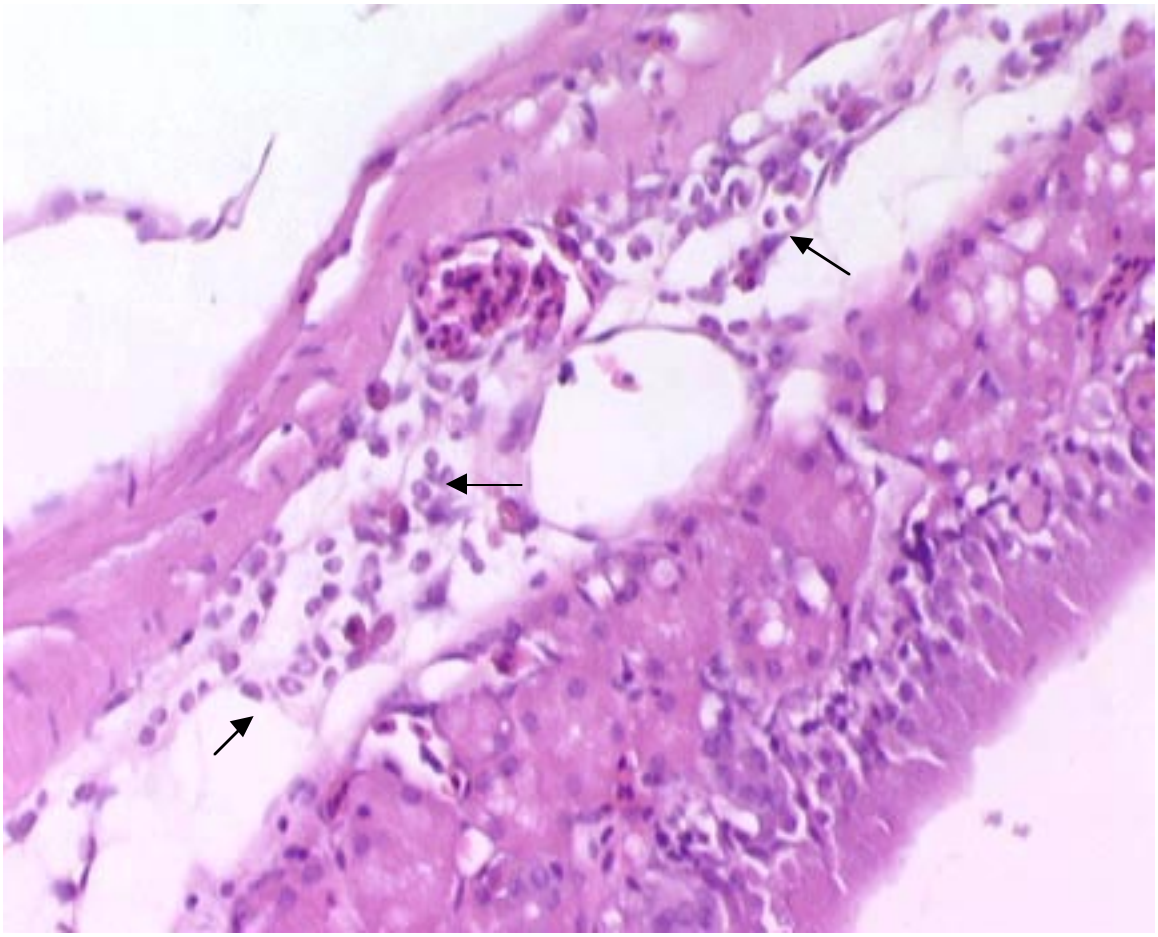

Figure 4-23. *Spironucleus vortens* (arrows) were occasionally observed within the gastric submucosa of an IP-infected angelfish (H&E, 20X).

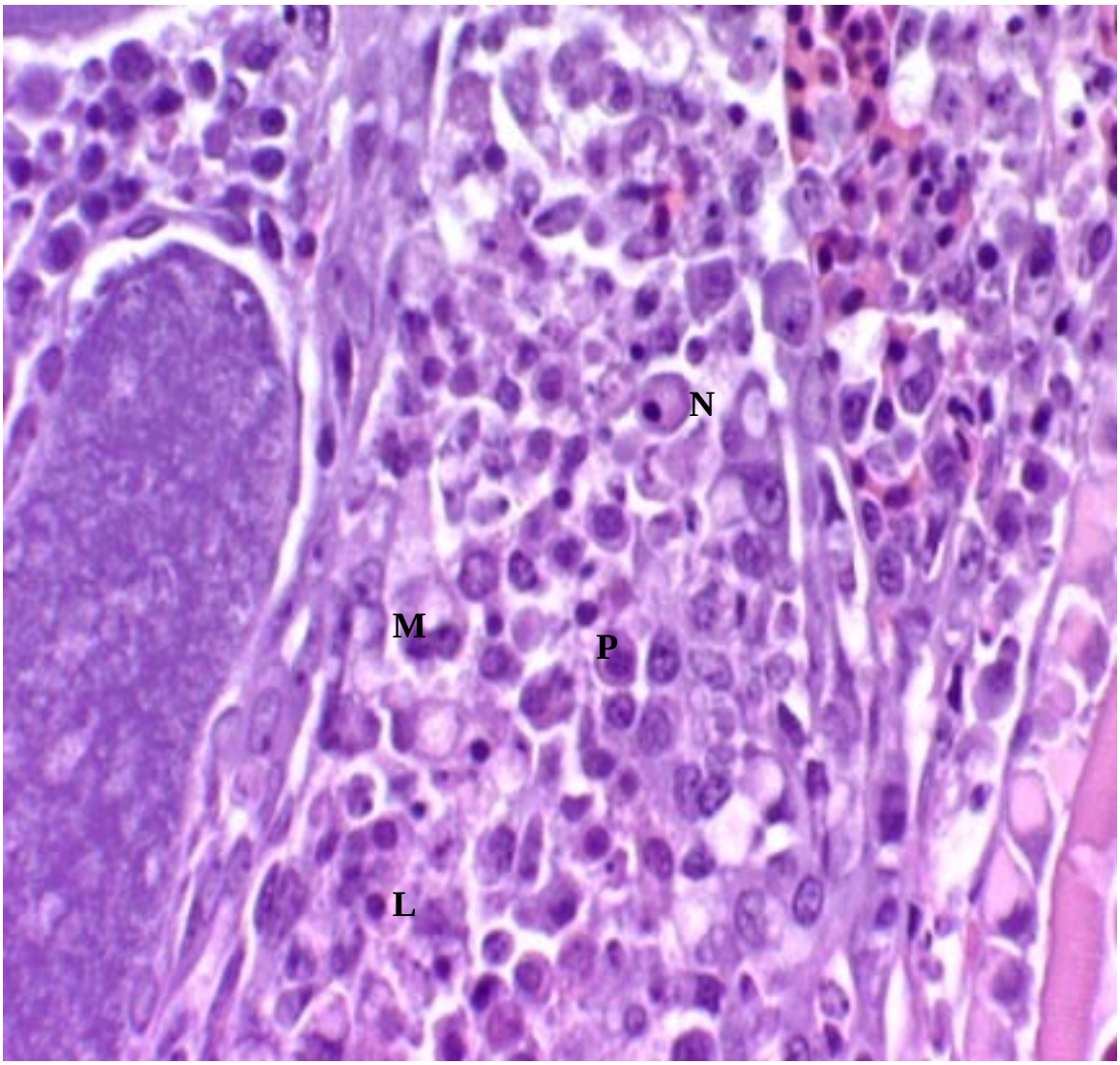

Figure 4-24. Granulomatous inflammation with infiltration of macrophages (M), lymphocytes (L), and plasma cells (P) in the interfollicular areas of the ovary. Individual cell necrosis (N) was also observed in these areas (H&E, 40X).

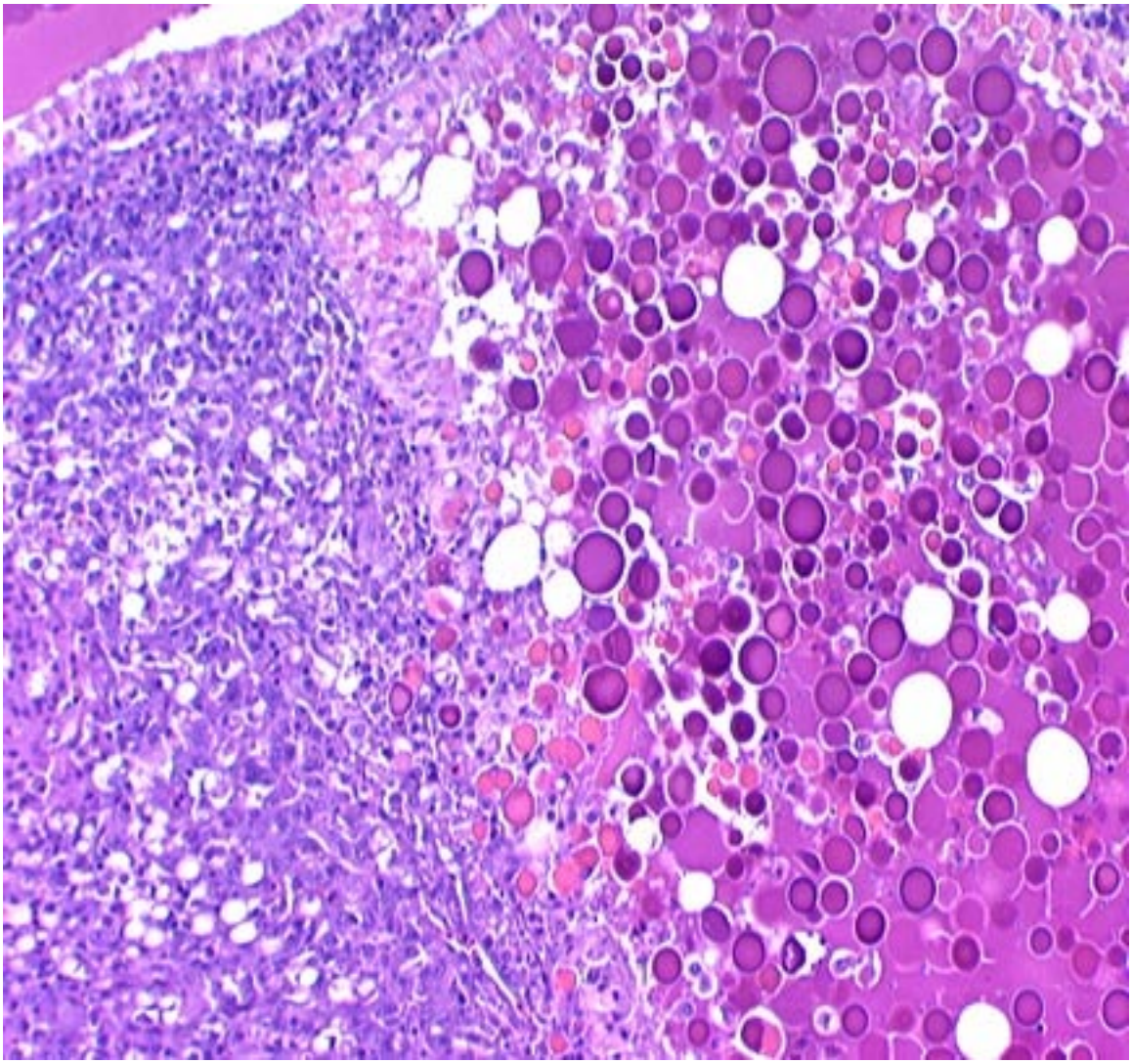

Figure 4-25. A ruptured follicle was observed in severe systemic infection of the angelfish (H&E, 20X).

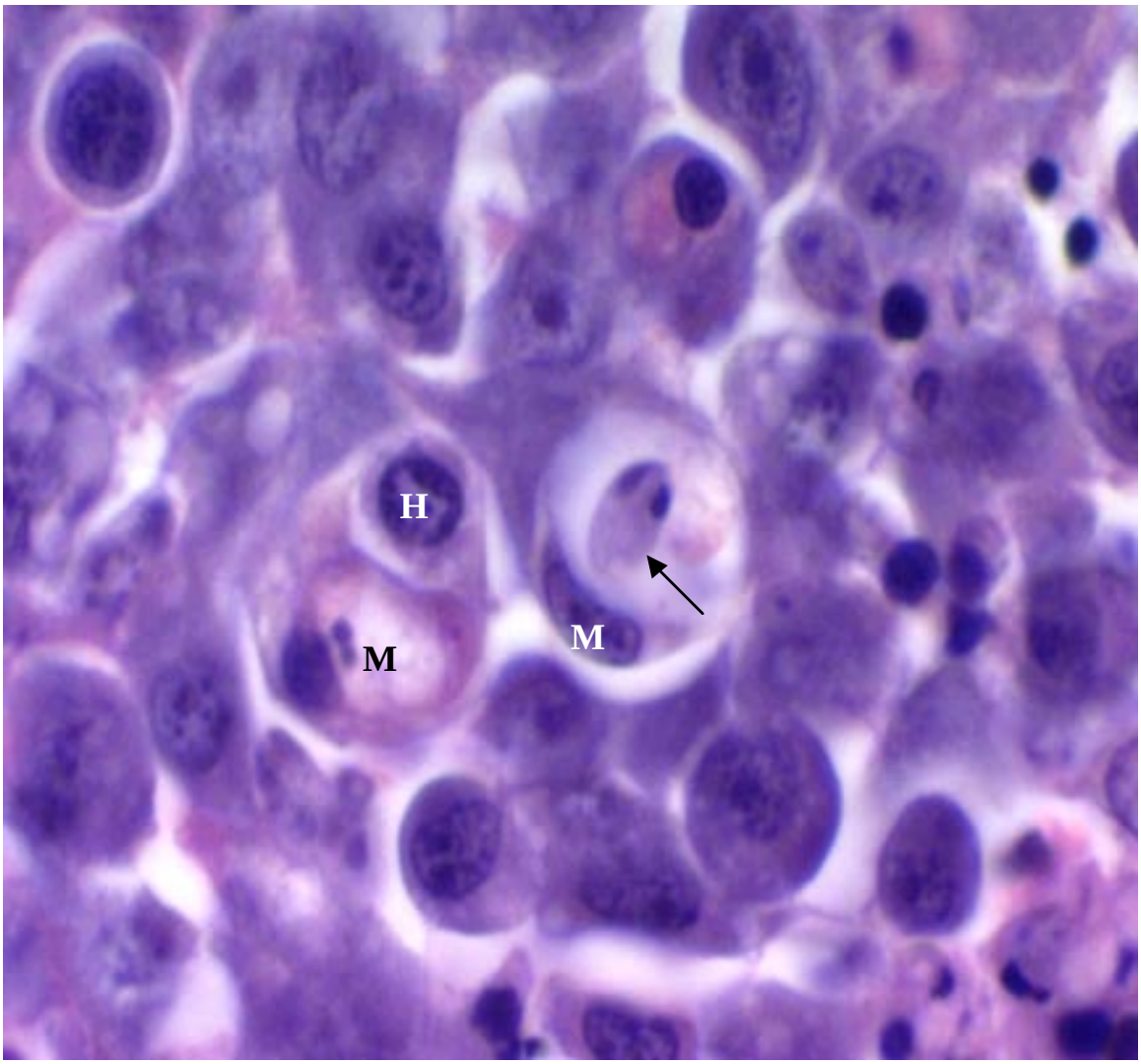

Figure 4-26. Macrophages (M) engulfing dead host cell (H) and *S. vortens* (arrow) were observed in the interfollicular interstitium in the ovary (H&E, 100X).

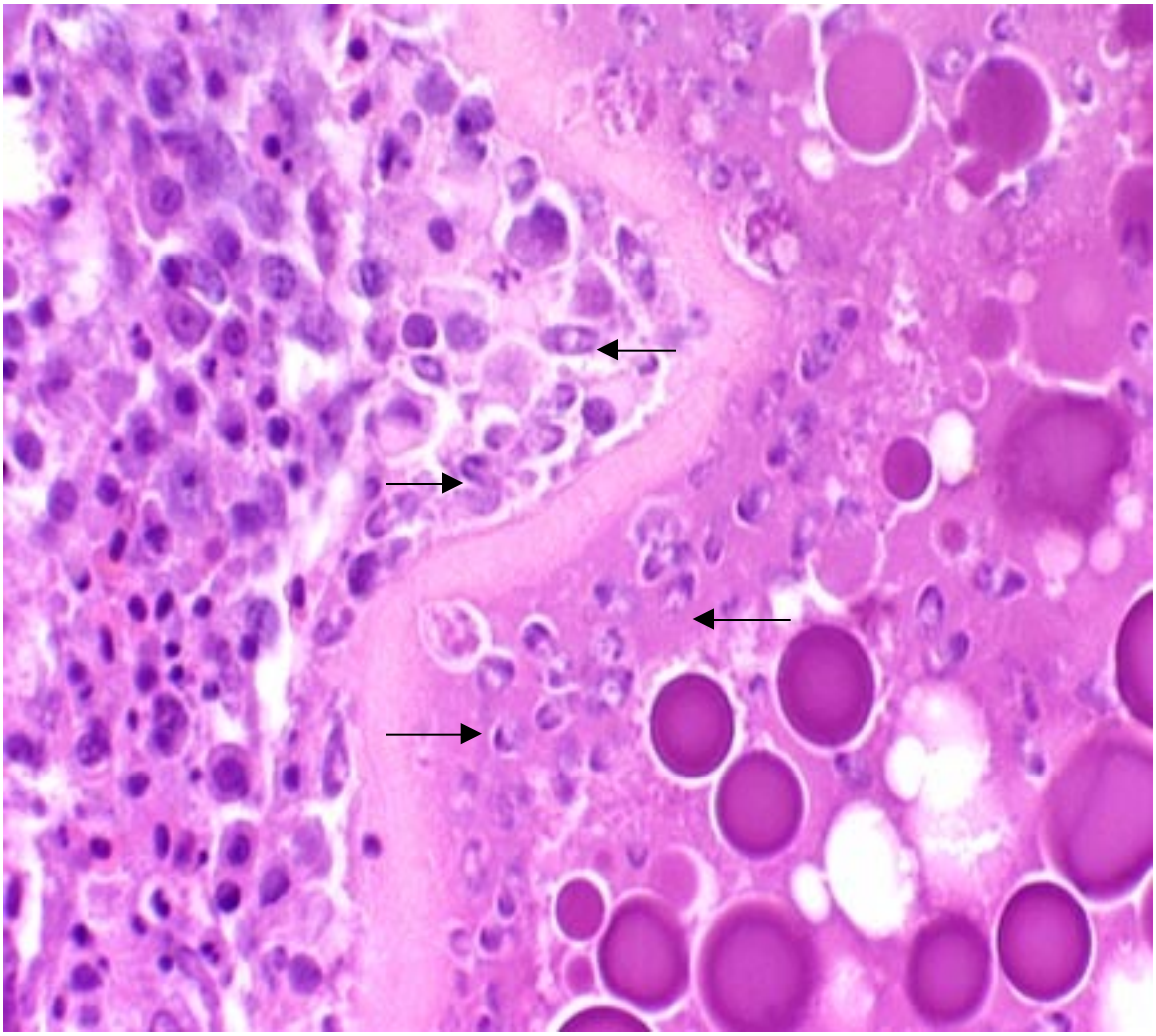

Figure 4-27. *Spironucleus vortens* (arrows) were abundant within the ovarium interstitium and also within the periphery of follicle (H&E, 40X).

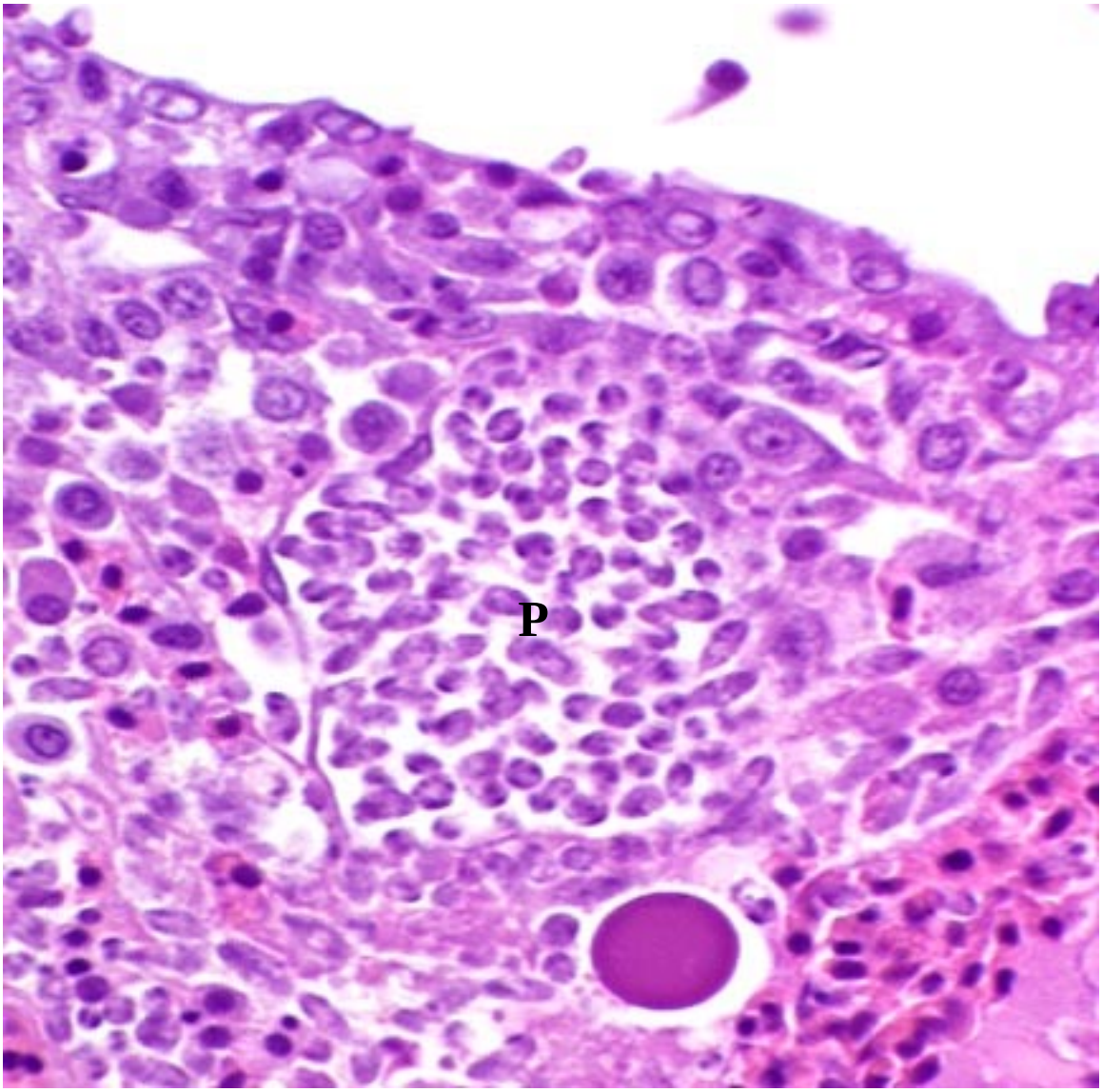

Figure 4-28. *Spironucleus vortens* (P) were found within a blood vessel in the interstitium of the ovary (H&E, 40X).

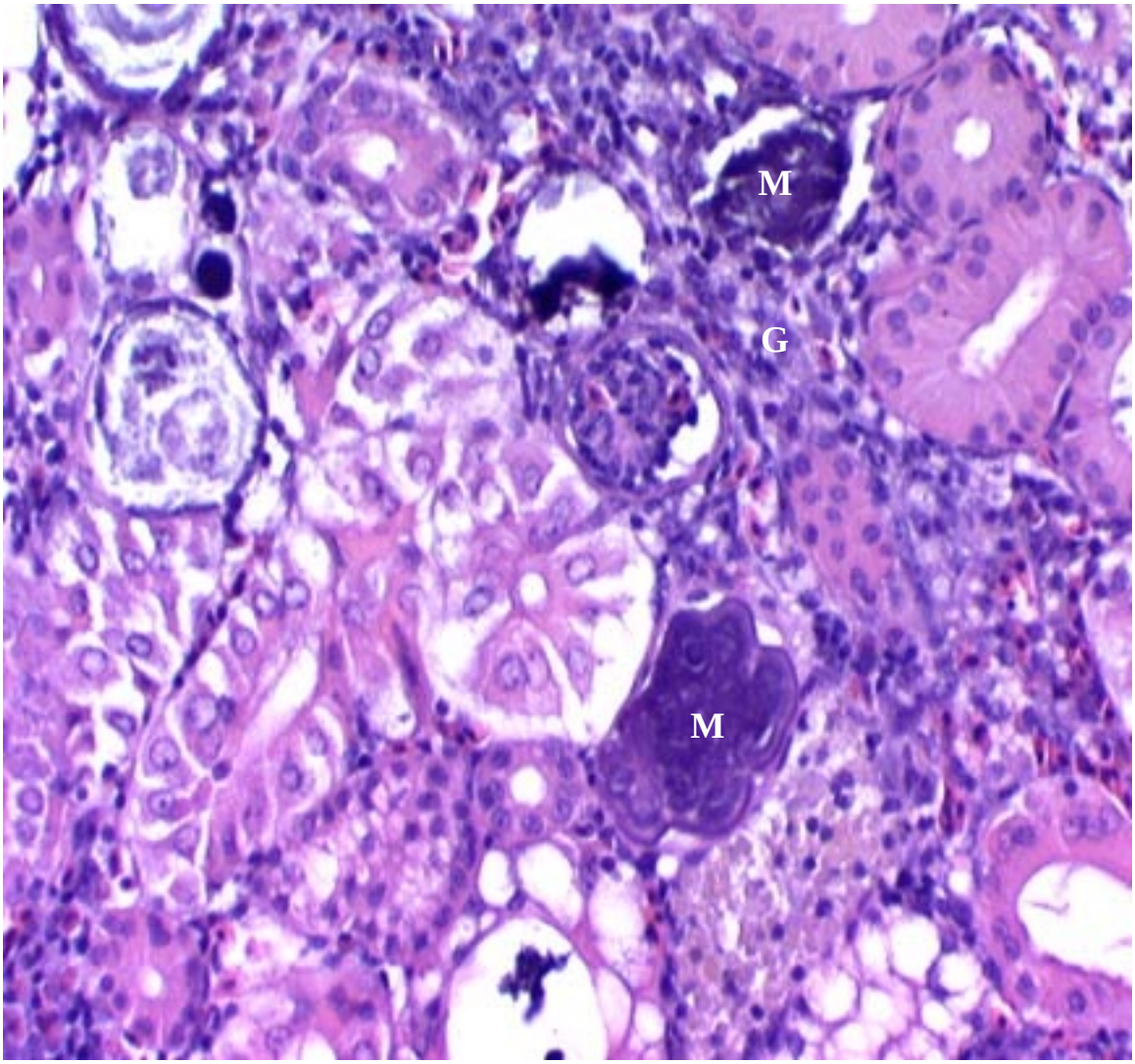

Figure 4-29. Extensive mineral deposition (M) and reactive granuloma formation (G) in the kidney of IP-infected angelfish (H&E, 20X).

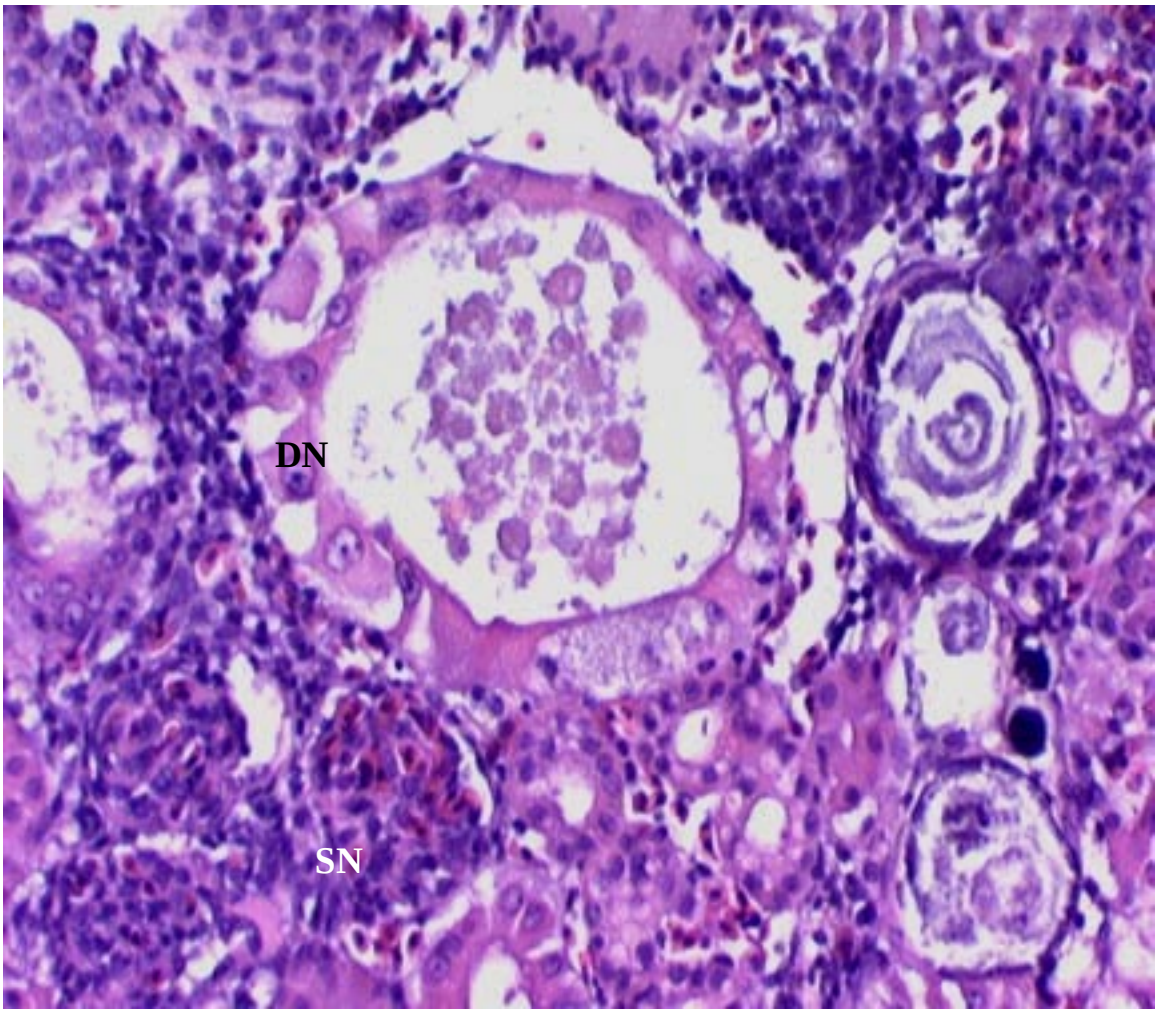

Figure 4-30. Pathological changes of renal tubules (DN = degeneration and necrosis) and glomeruli (SN = synechia) in the kidney of IP-infected angelfish (H&E, 20X).

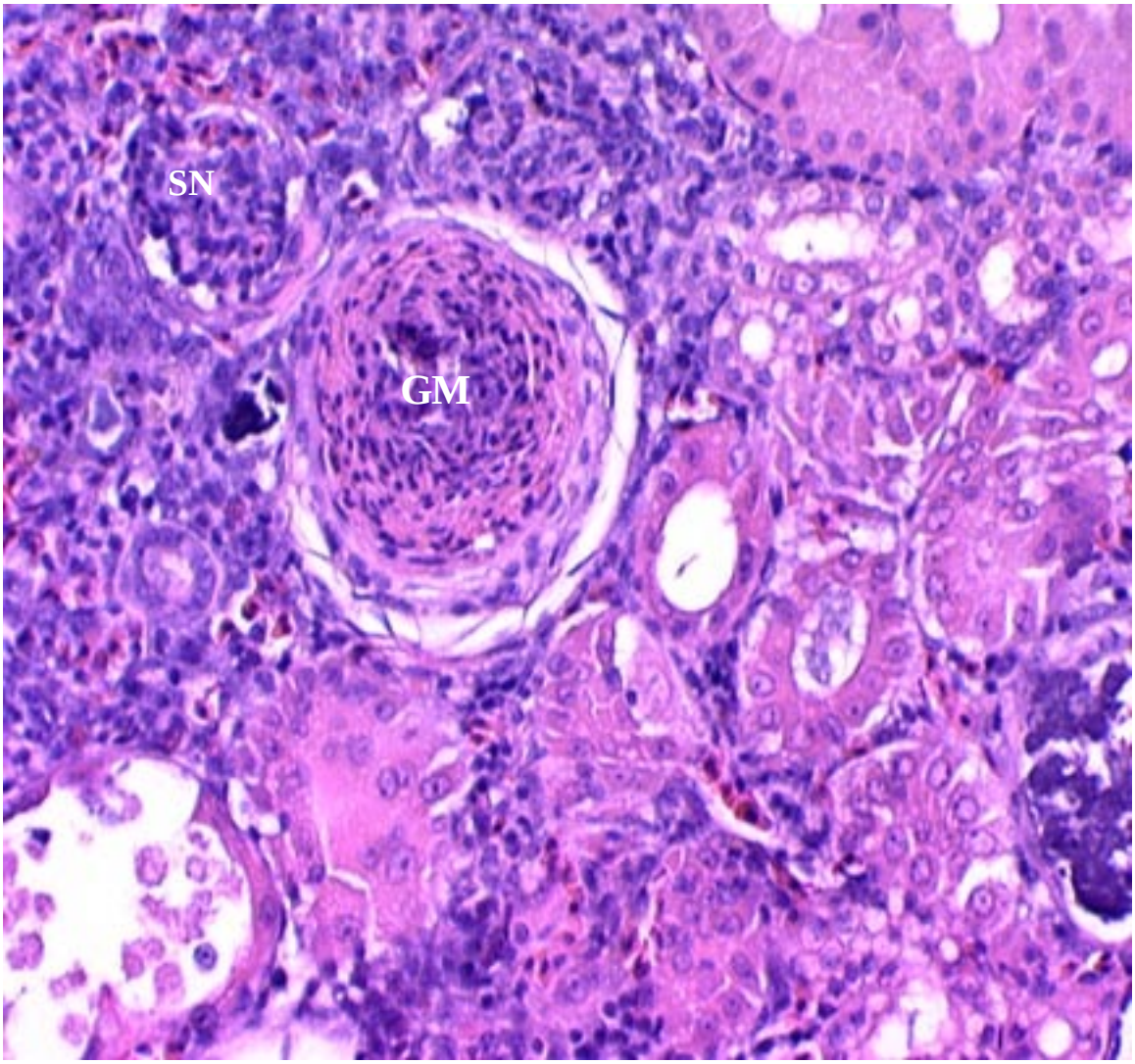

Figure 4-31. Pathological changes in the glomeruli included the presence of increased mesangial matrix, synechia (SN), and glomerulosclerosis (GM) of IP-infected angelfish (H&E, 40X).

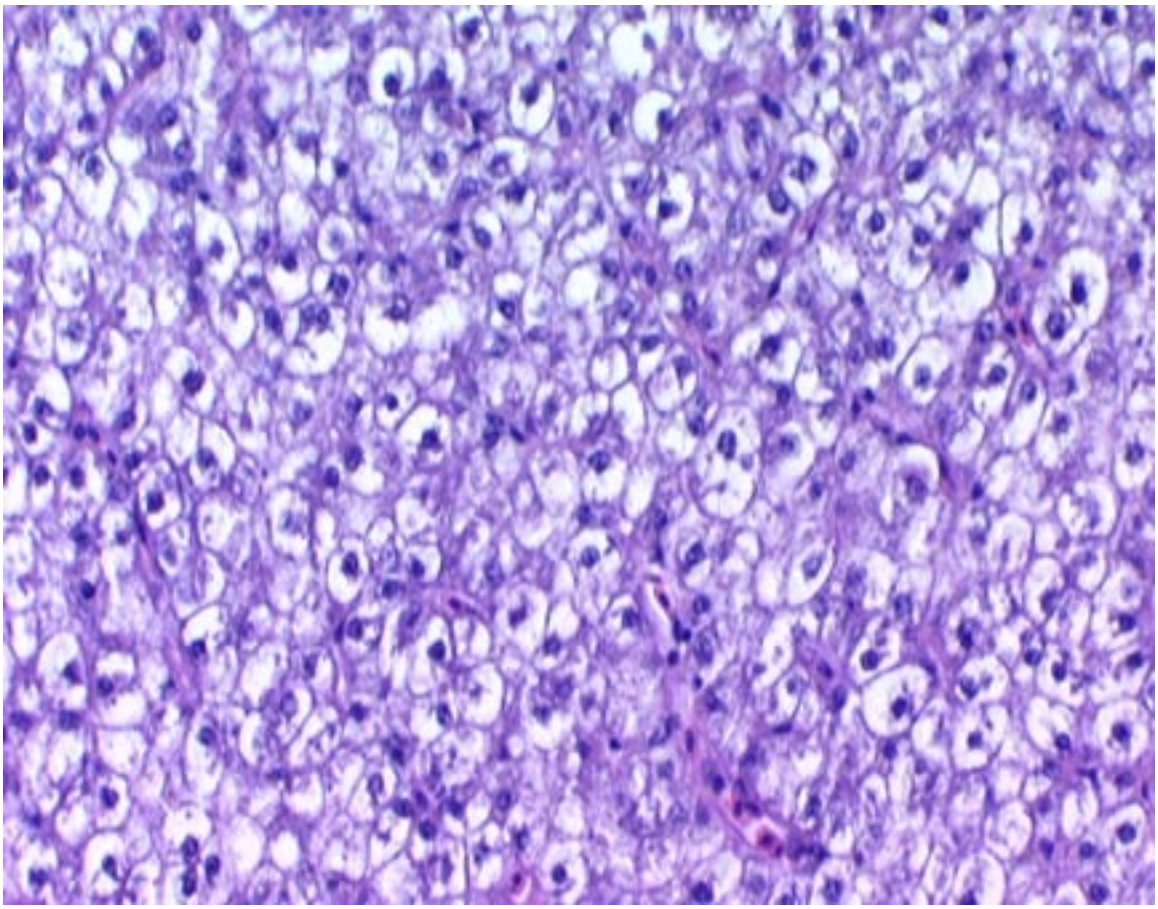

Figure 4-32. General appearance of normal hepatopancreas of a control angelfish (H&E, 20X).

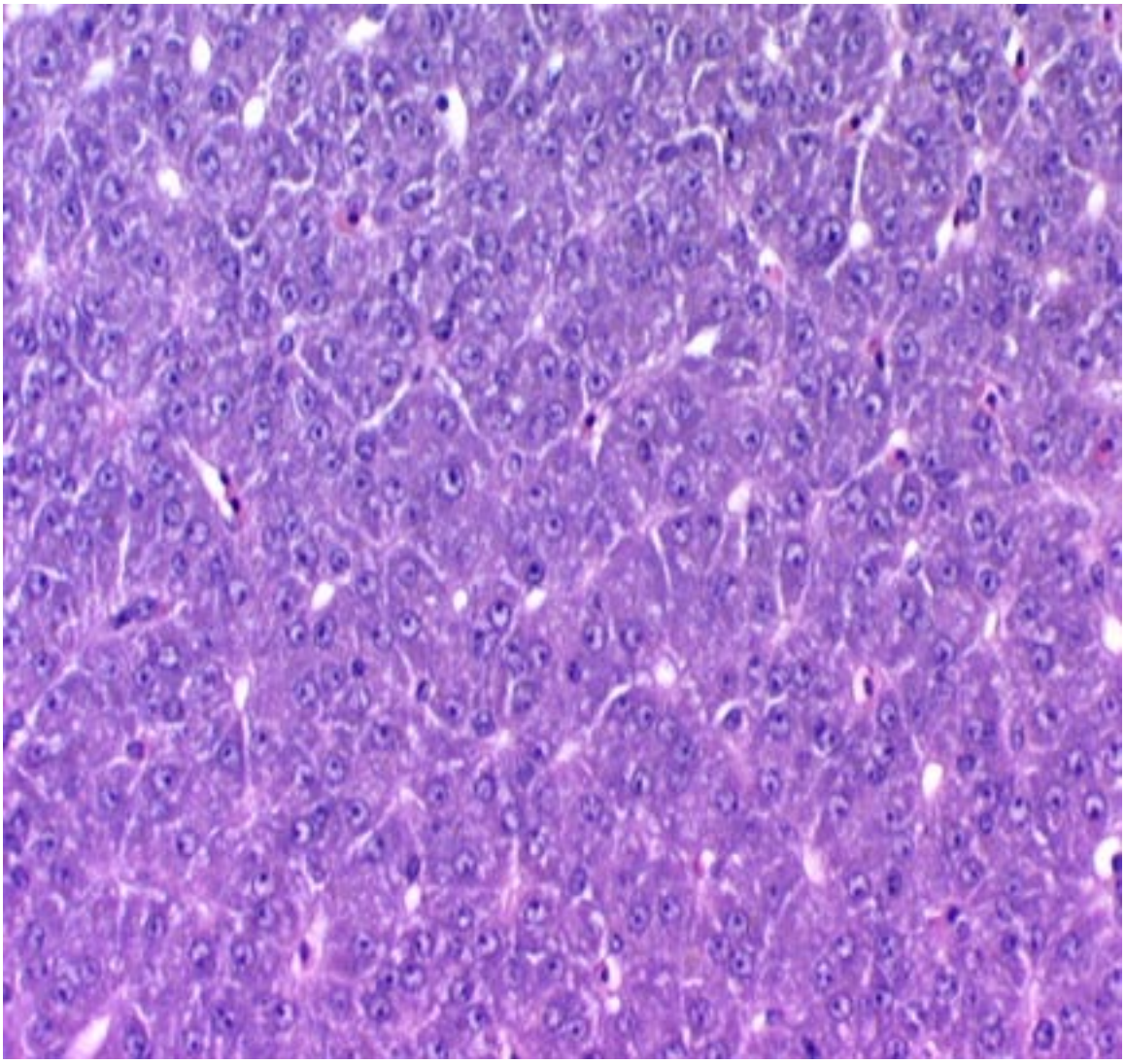

Figure 4-33. Decreased hepatocyte vacuolization indicating a status of fat and glycogen depletion of infected angelfish (H&E, 20X).

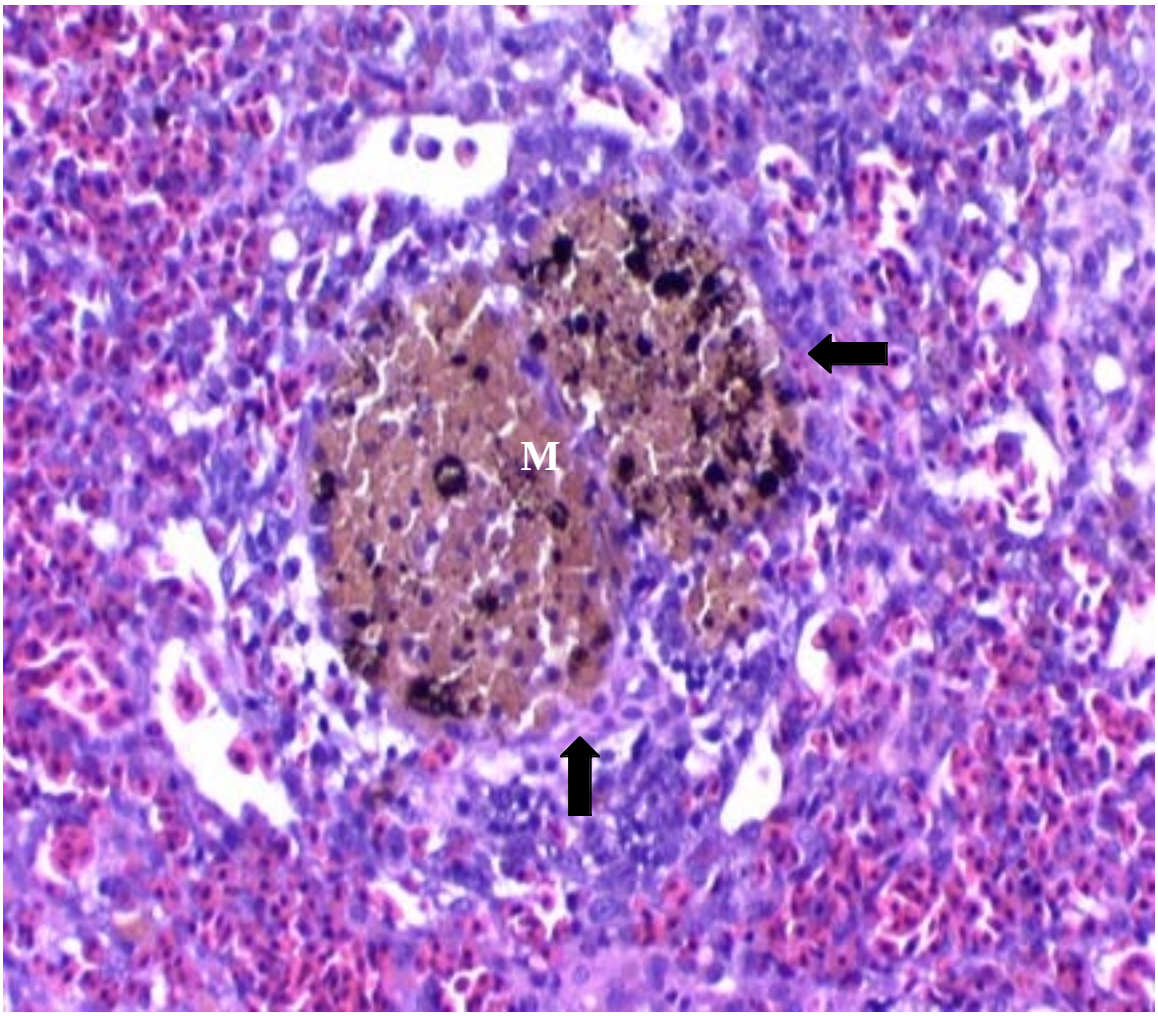

Figure 4-34. Mild fibrosis (arrows) at the perimeter of melano-macrophage (M) center in the spleen of IP-infected angelfish (H&E, 20X).

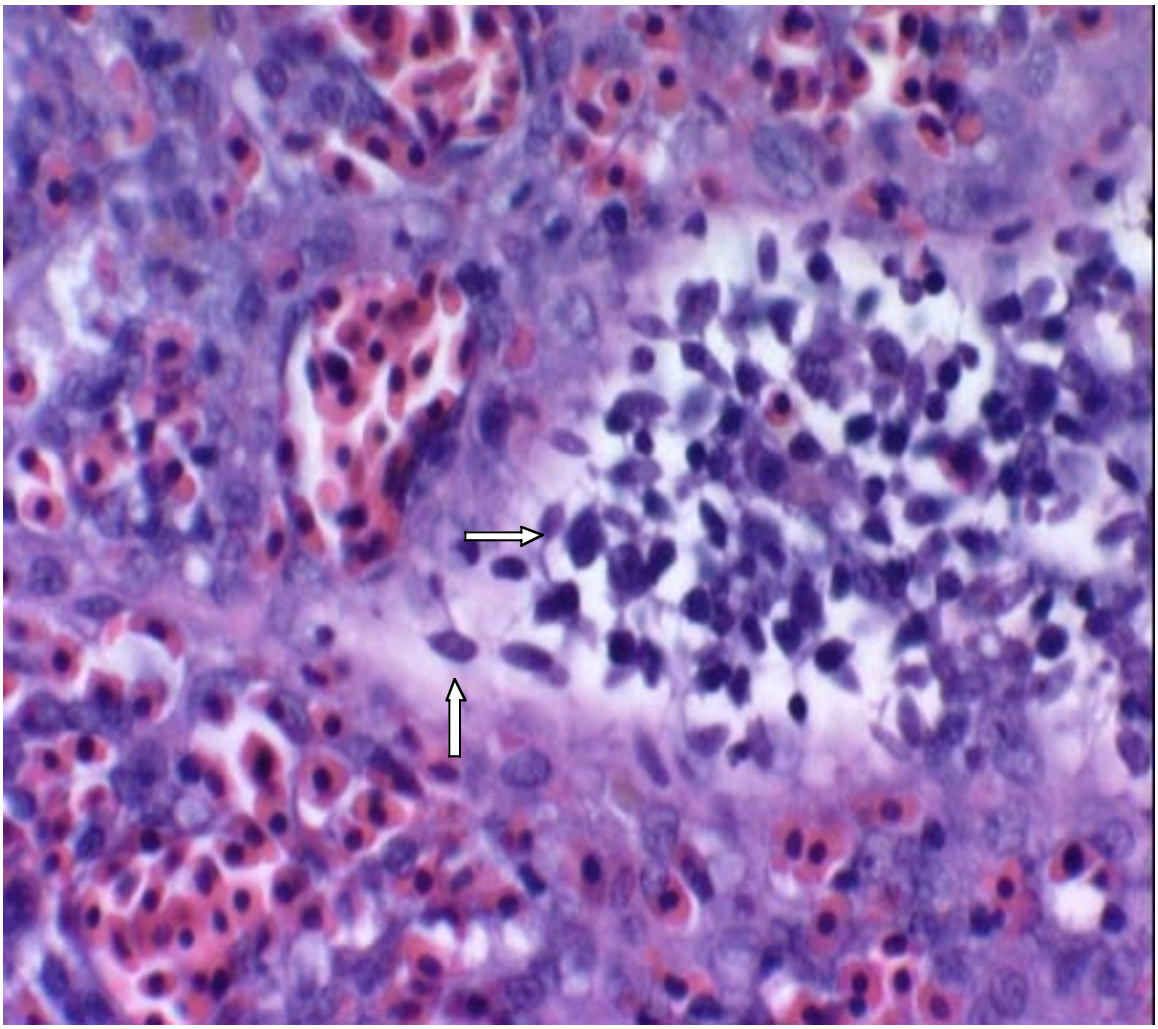

Figure 4-35. *Spironucleus vortens* (arrows) as observed in the spleen of IP-infected angelfish (H&E, 40X).

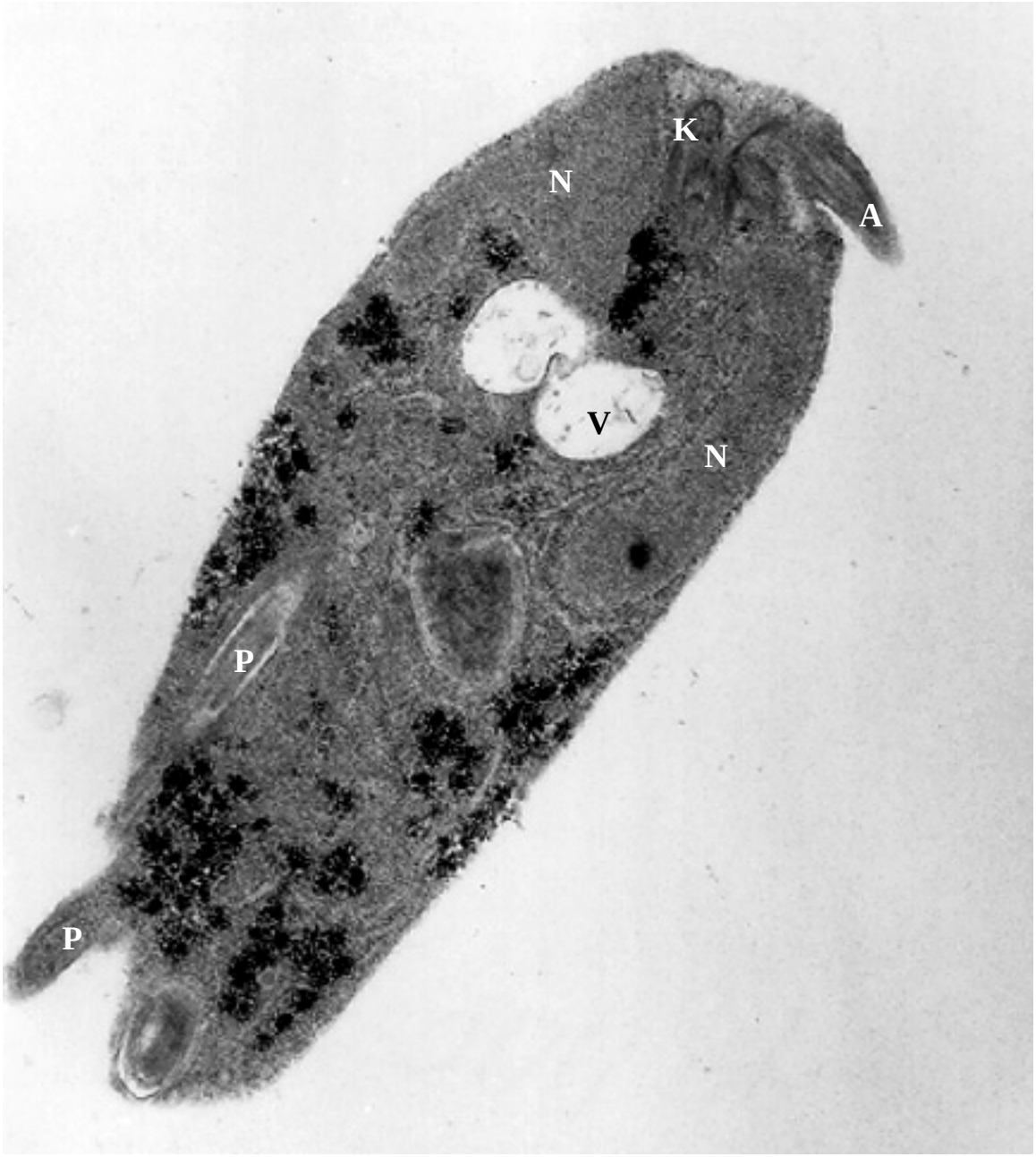

Figure 4-36. Ultrastructures of *S. vortens* viewed under transmission electron microscope (TEM) (19,440X). A = anterior flagellum, K = kinetosome, N = nucleus, P = recurrent posterior flagellum, V = vacuole

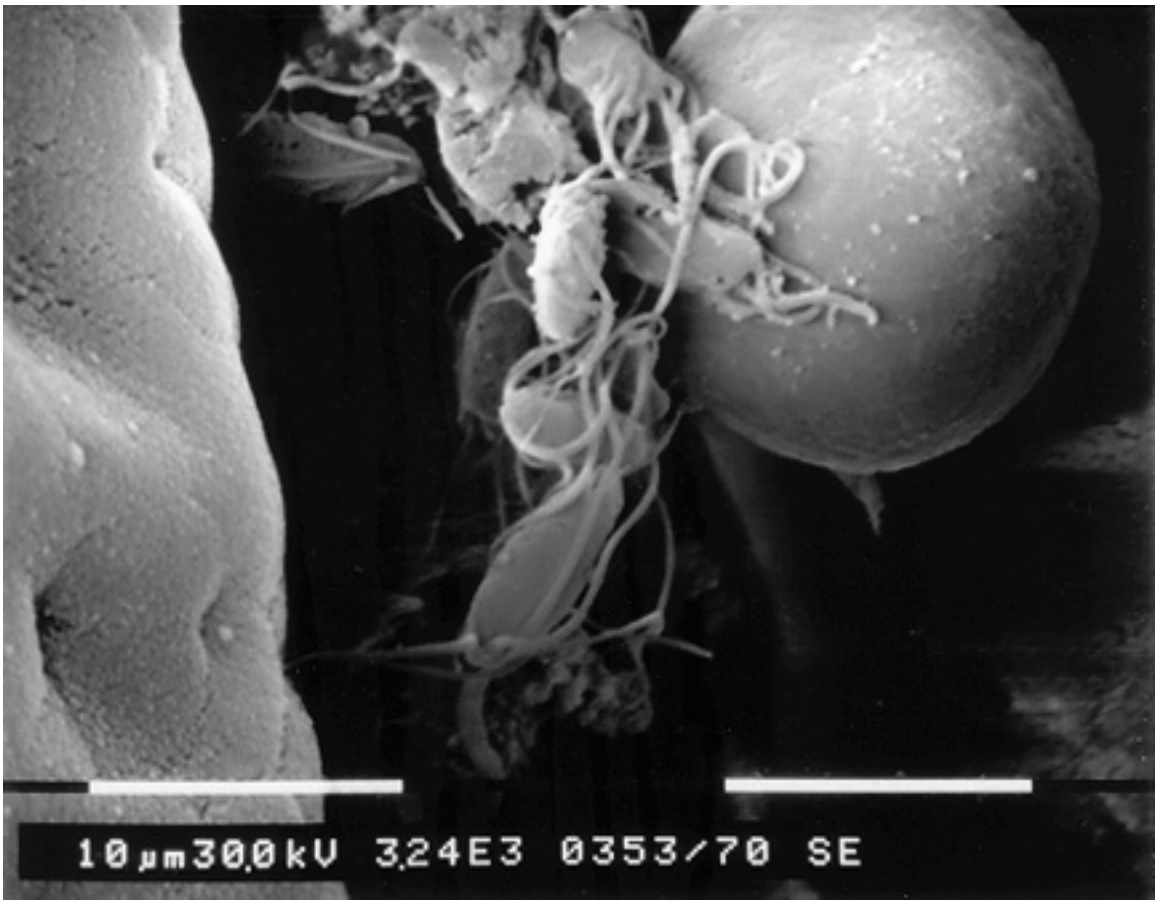

Figure 4-37. *Spironucleus vortens* found adjacent to the intestinal mucosa in the angelfish (3,240X, SEM).

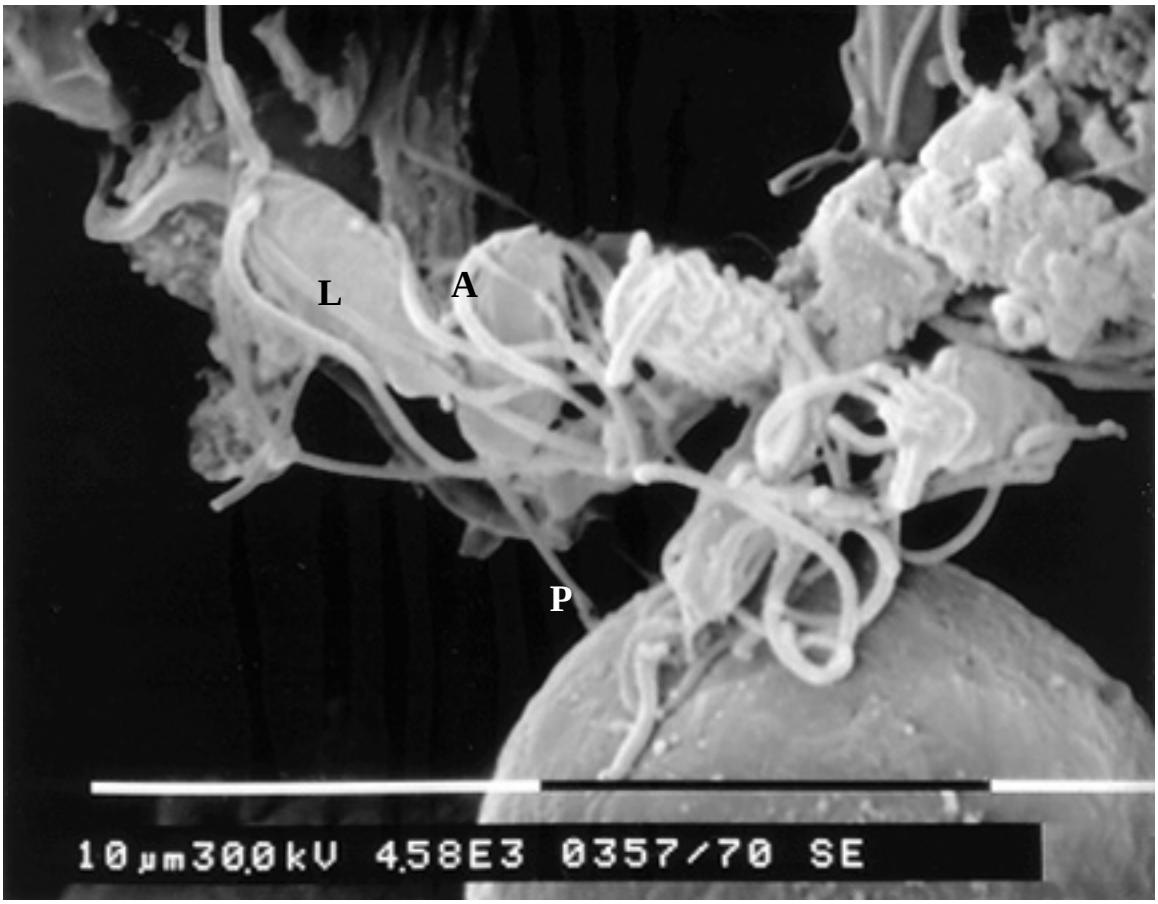

Figure 4-38. *Spironucleus vortens* from the angelfish with anterior (A) and posterior (P) flagella, and lateral longitudinal ridges (4,580X, SEM).

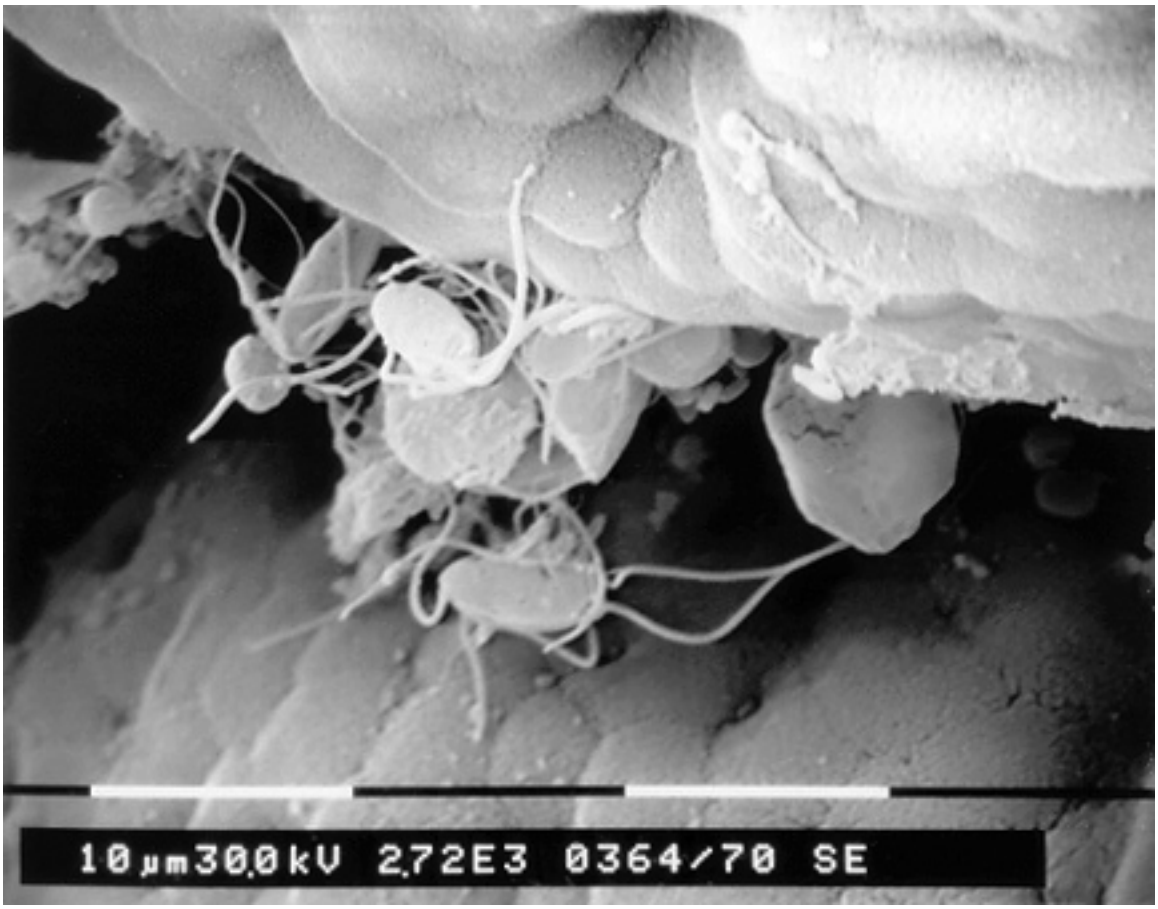

Figure 4-39. *Spironucleus vortens* from the angelfish which appears attached to the intestinal mucosa (2,7200X, SEM)

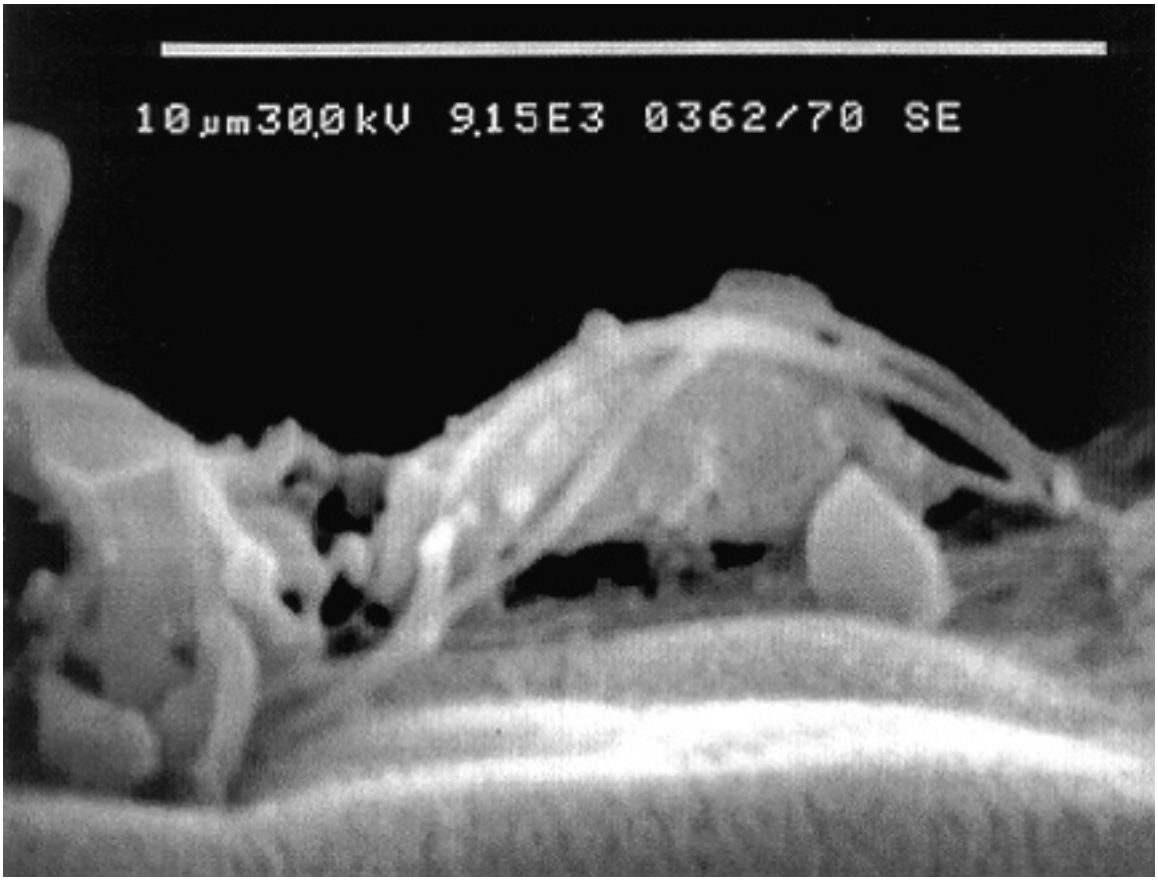

Figure 4-40. *Spironucleus vortens* were found attach to the intestinal mucosa but without invading the tissue (9,150X, SEM).

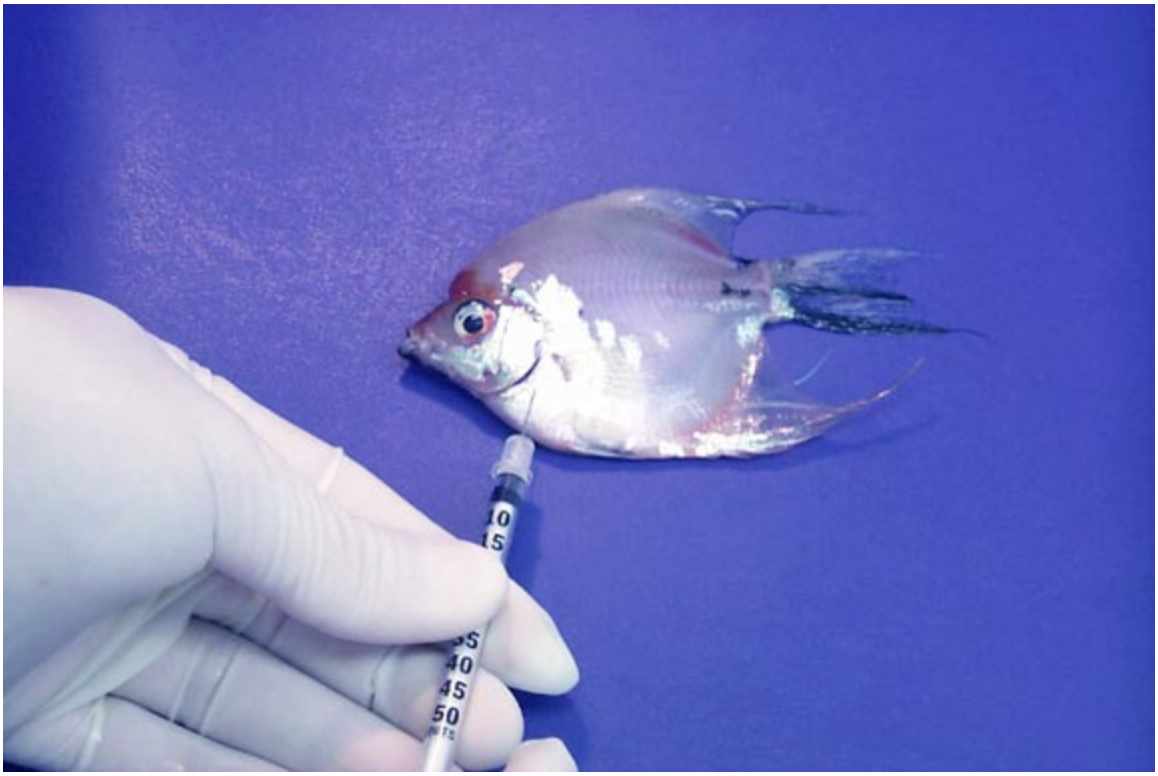

Figure 5-1. Position to collect blood from the angelfish’s heart.

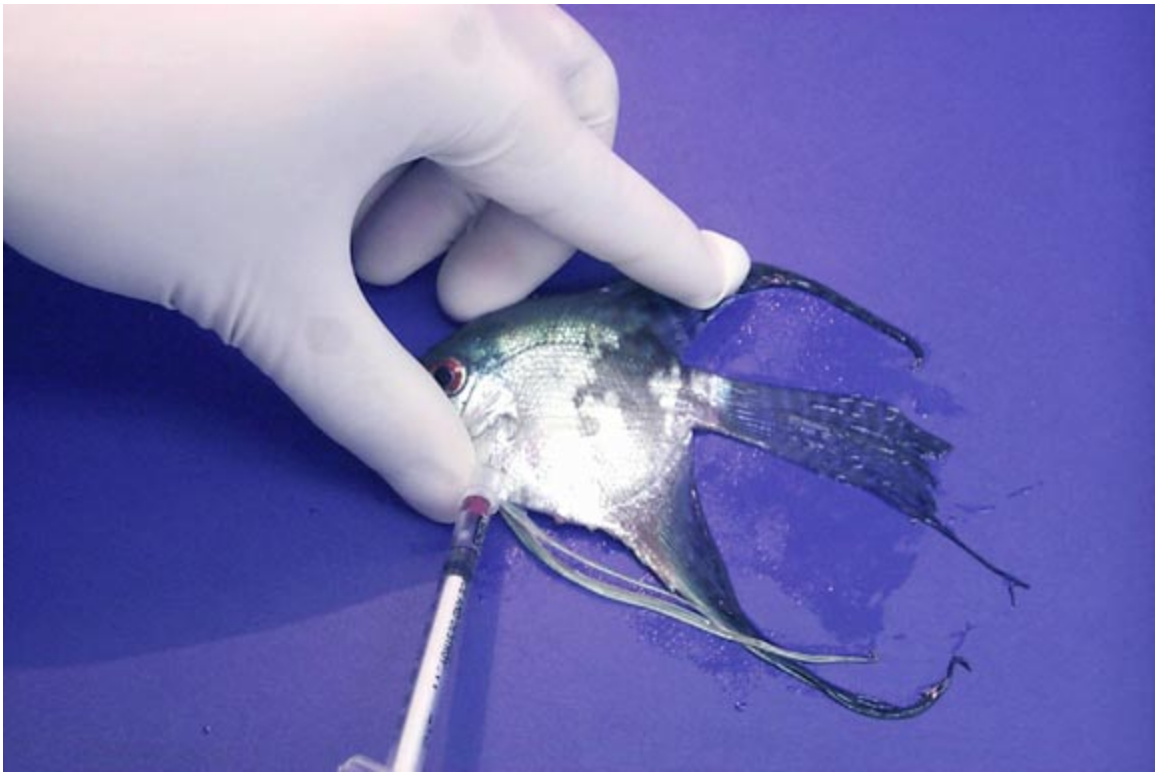

Figure 5-2. Blood collecting (heart puncture) in anesthetized angelfish.

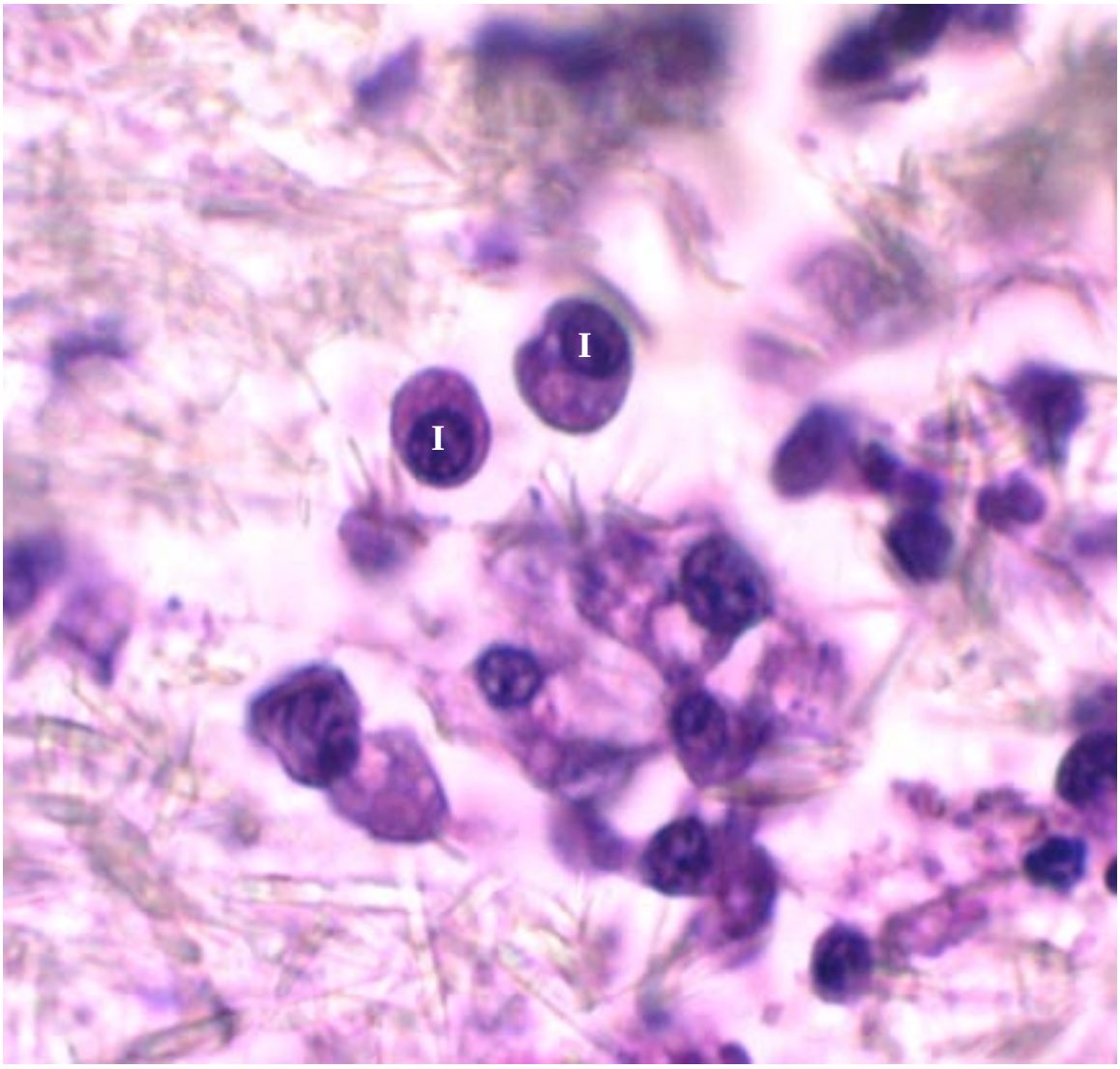

Figure 5-3. Inflammatory cells (I) infiltrated into the peritoneum (H&E, 100X).

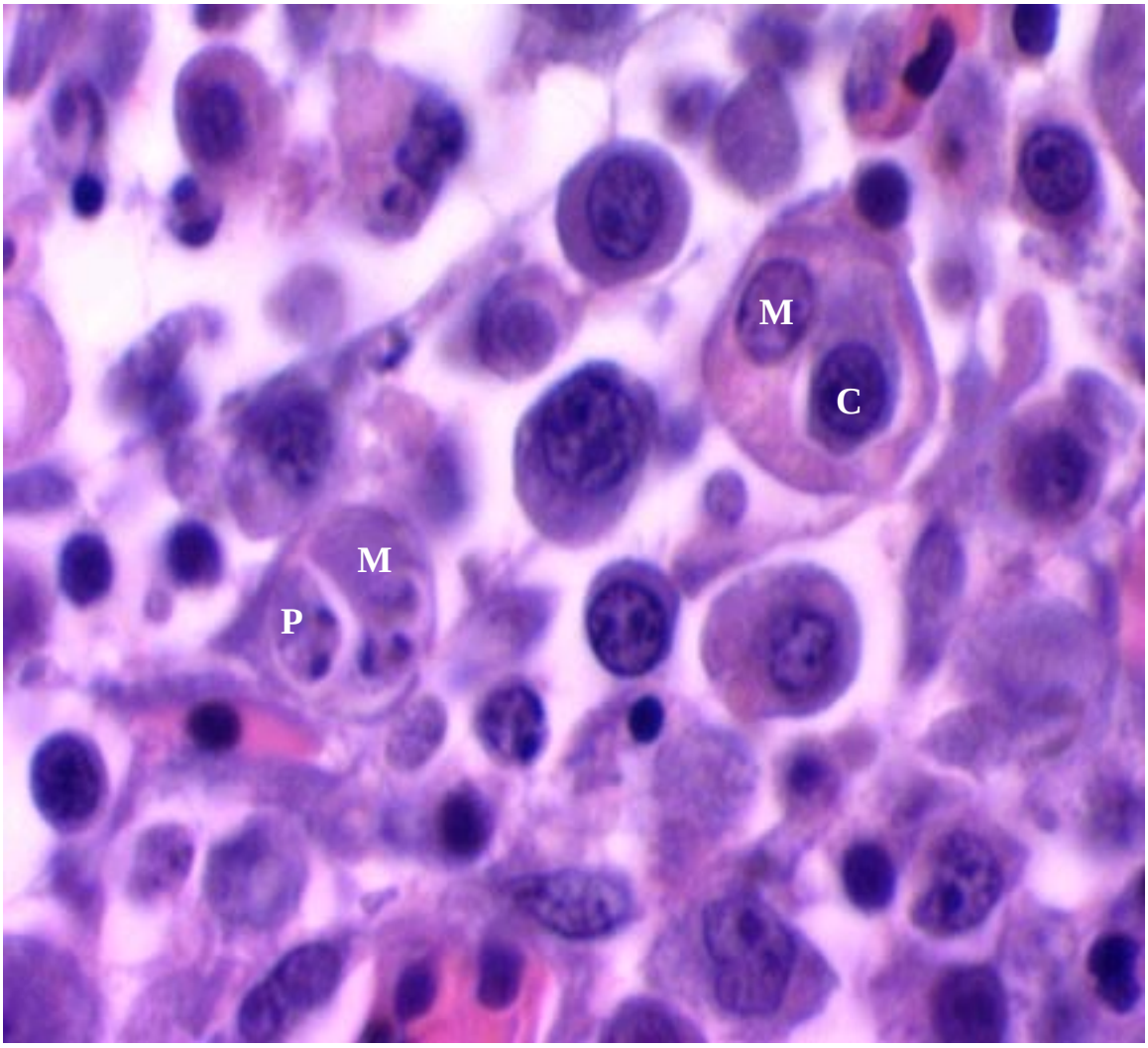

Figure 5-4. Macrophages (M) with engulfed parasites (P) and other cells (C) (H&E, 100X).

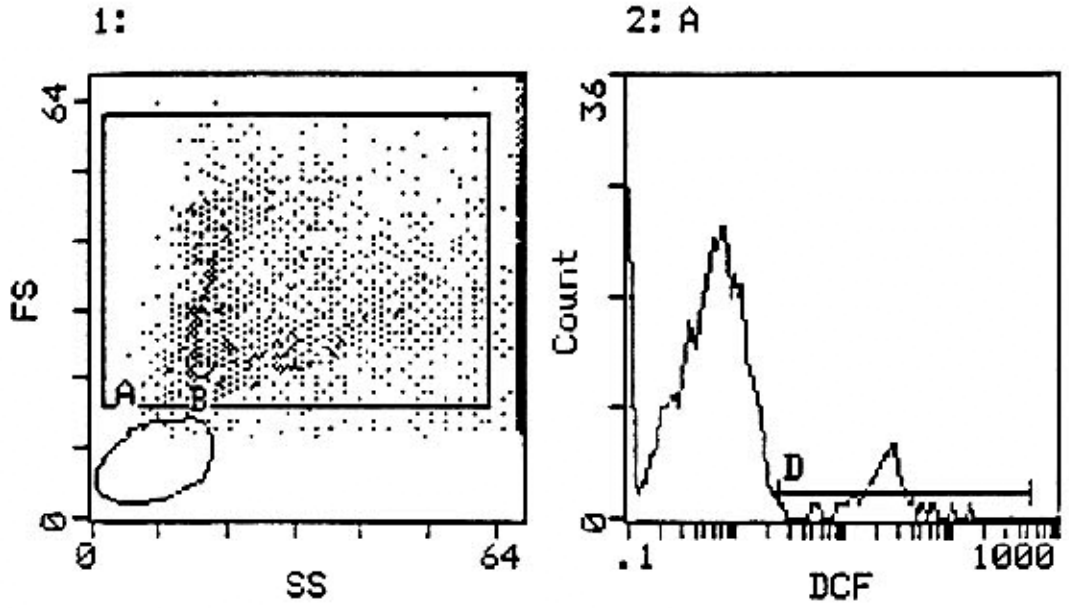

Figure 5-5. Electronic measurement of dichlorofluorescein (DCF) in kidney macrophages using a flow cytometer (FS = forward scatter, SS = side scatter, DCF = dichlorofluorescein)

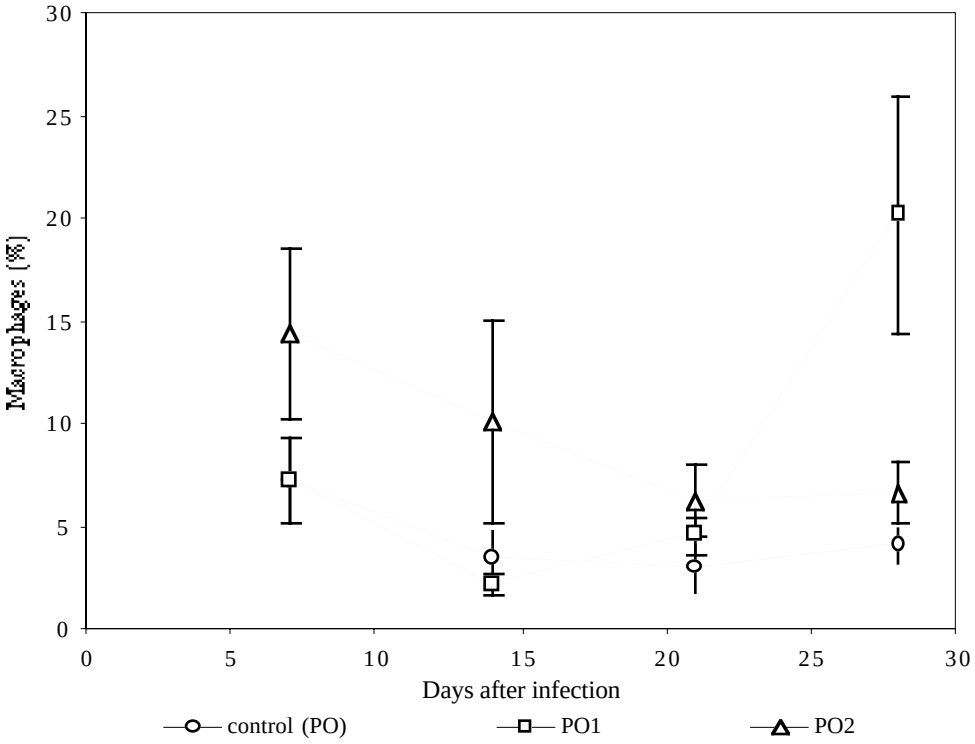

Figure 5-6. Percentage of macrophages producing H_2O_2 in angelfish orally infected with two different doses of *S. vortens* (Bars indicated standard error of mean)

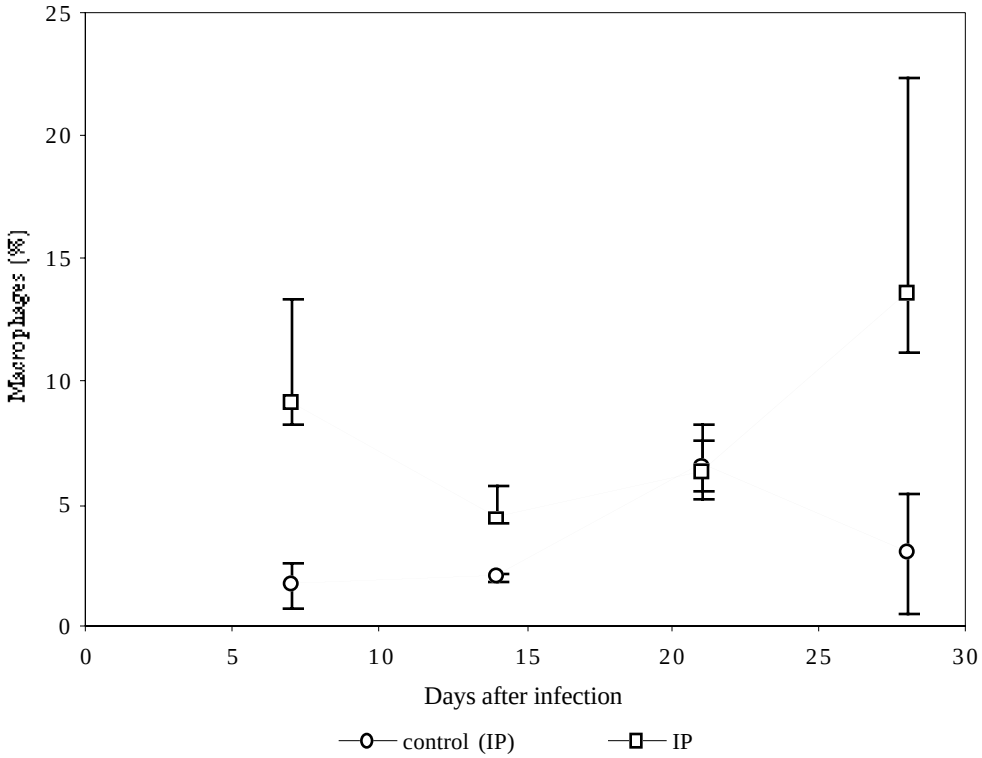

Figure 5-7. Percentage of macrophages producing H_2O_2 in angelfish IP-infected with *S. vortens* (Bars indicated standard error of mean)

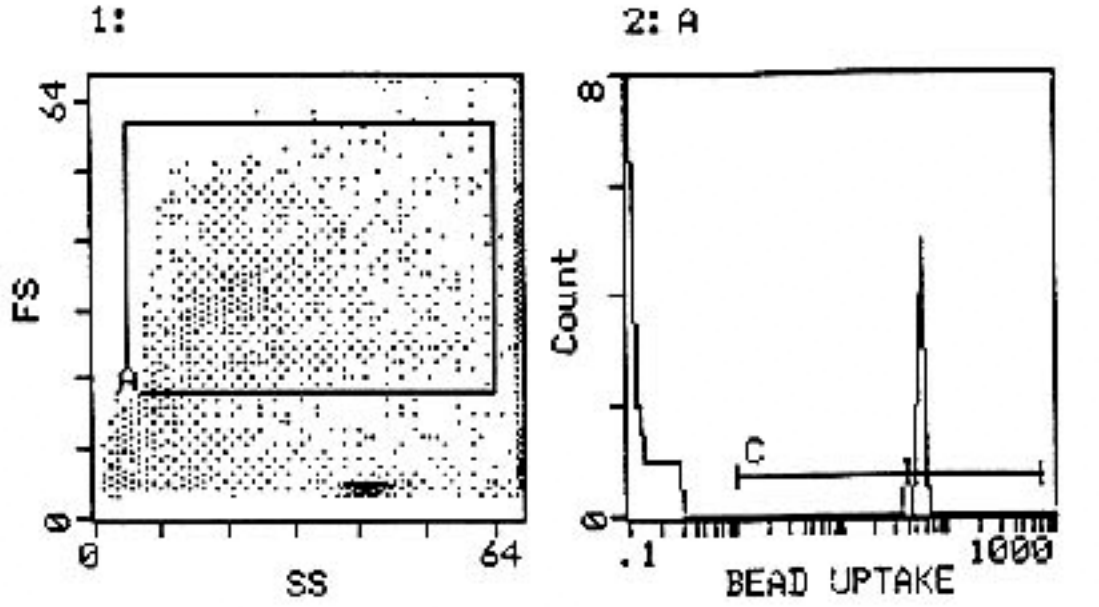

Figure 5-8. Electronic measurement of fluoresbrite-microsphere uptake in macrophages using a flow cytometer (FS = forward scatter, SS = side scatter).

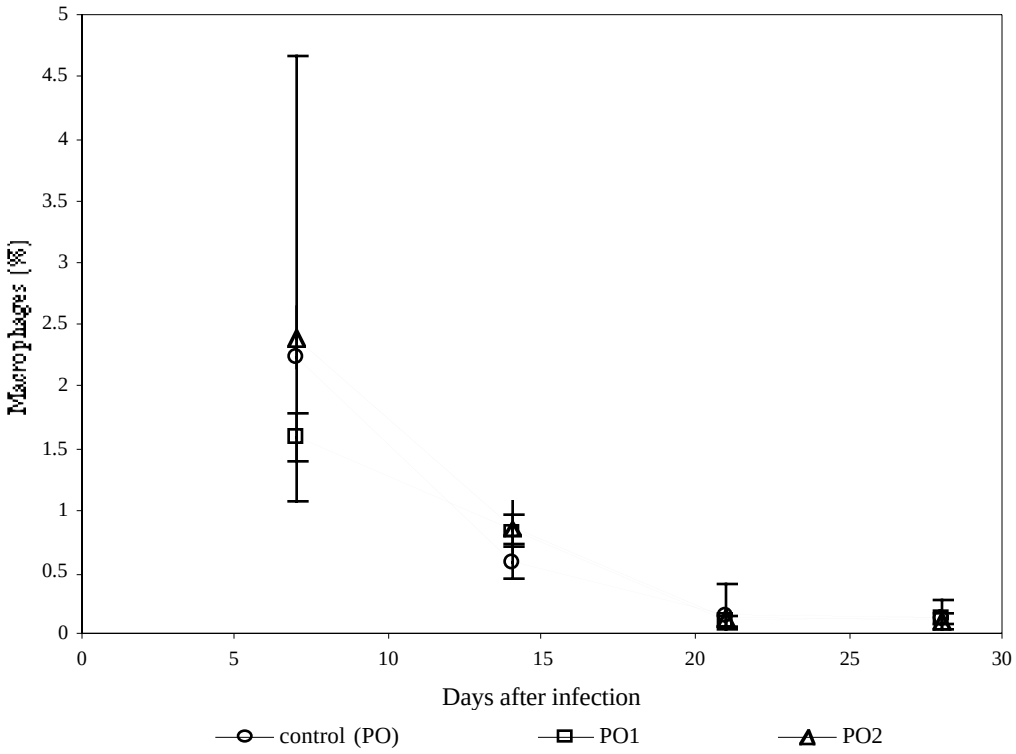

Figure 5-9. Percentage of phagocytic macrophages in angelfish orally infected with two different doses of *S. vortens* (Bars indicated standard error of mean)

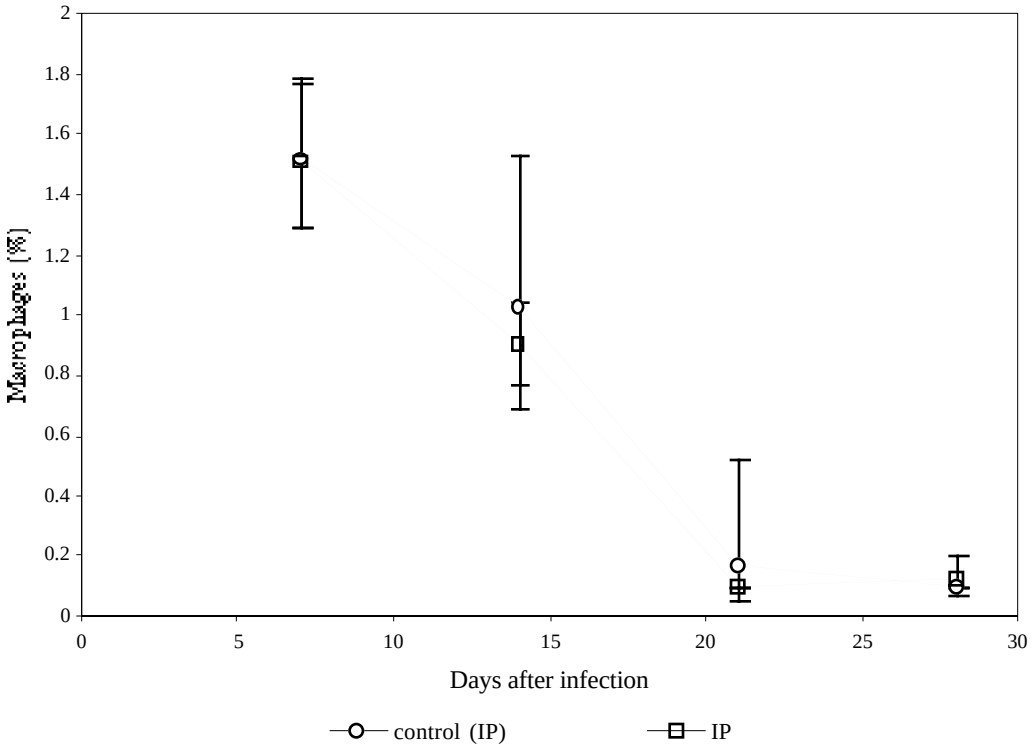

Figure 5-10. Percentage of phagocytic macrophages in angelfish IP-infected with *S. vortens (* Bars indicated standard error of mean)

TABLES

Table 2-1. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated at different temperatures

	Temperature (°C)					
day	22	25	28	31	34	37
1	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00
2	0.073 ± 0.01	0.189 ± 0.01	0.201 ± 0.02	0.161 ± 0.01	0.094 ± 0.01	0
3	0.359 ± 0.12	2.010 ± 0.38	1.939 ± 0.24	2.183 ± 0.58	0.320 ± 0.04	0
4	1.591 ± 0.65	5.813 ± 0.48	7.919 ± 0.48	6.659 ± 0.63	0.730 ± 0.17	0
5	4.520 ± 0.67	5.999 ± 0.49	0	0	0	0
6	5.718 ± 0.29	5.672 ± 0.37	0	0	0	0
7	5.767 ± 0.35	0	0	0	0	0
8	5.999 ± 0.32	0	0	0	0	0
9	5.801 ± 0.22	0	0	0	0	0
10	5.758 ± 0.24	0	0	0	0	0

Table 2-2. Average cell number of *S. vortens* (million cells ml^{-1}) from day 2 to day 4 of cultures at different temperatures*

	Temperature (°C)				
day	22	25	28	31	34
2	0.072^{a}	0.188^{b}	0.200^{b}	0.161^{b}	0.094^{a}
3	0.345^{a}	1.987^{b}	1.929^{b}	2.127^{b}	0.318^{a}
4	1.497^{a}	5.800^{b}	7.910^{b}	6.639^{b}	0.718^{c}

*Back-transformed means within a row (day) followed by different letters are significantly different at $\alpha = 0.05$ according to Turkey's HSD

Table 2-3. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated at different pH conditions

	pH												
day	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
1	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00
2	0.030 ± 0.00	0.034 ± 0.00	0.032 ± 0.00	0.038 ± 0.00	0.043 ± 0.00	0.060 ± 0.00	0.050 ± 0.00	0.036 ± 0.00	0.041 ± 0.01	0.033 ± 0.01	0.043 ± 0.00	0.042 ± 0.00	0.040 ± 0.01
3	0.098 ± 0.01	0.122 ± 0.00	0.105 ± 0.01	0.171 ± 0.02	0.215 ± 0.01	0.953 ± 0.05	0.515 ± 0.03	0.165 ± 0.01	0.184 ± 0.01	0.108 ± 0.01	0.143 ± 0.01	0.104 ± 0.00	0.102 ± 0.01
4	0.142 ± 0.02	0.167 ± 0.00	0.134 ± 0.01	0.211 ± 0.02	2.377 ± 0.14	6.456 ± 0.20	6.085 ± 0.22	0.894 ± 0.04	0.291 ± 0.02	0.182 ± 0.01	0.231 ± 0.00	0.130 ± 0.01	0.120 ± 0.01
5	0	0	0	0.156 ± 0.01	4.646 ± 0.07	11.127 ± 0.14	10.770 ± 0.47	6.609 ± 0.21	0.227 ± 0.01	0.152 ± 0.00	0	0	0
6	0	0	0	0.124 ± 0.01	4.718 ± 0.31	10.600 ± 0.39	10.390 ± 0.33	11.707 ± 0.61	0.203 ± 0.02	0	0	0	0
7	0	0	0	0	4.461 ± 0.16	9.689 ± 0.19	10.277 ± 0.31	12.083 ± 0.69	0.143 ± 0.03	0	0	0	0
8	0	0	0	0	4.233 ± 0.13	9.257 ± 0.32	4.046 ± 0.10	8.063 ± 0.25	0	0	0	0	0
9	0	0	0	0	3.742 ± 0.61	1.225 ± 0.36	0.108 ± 0.05	0.083 ± 0.05	0	0	0	0	0
10	0	0	0	0	2.850 ± 0.03	0.150 ± 0.00	0.038 ± 0.00	0.025 ± 0.00	0	0	0	0	0
11	0	0	0	0	2.592 ± 0.35	0.083 ± 0.03	0.012 ± 0.01	0.003 ± 0.00	0	0	0	0	0
12	0	0	0	0	0.455 ± 0.05	0.026 ± 0.01	0.009 ± 0.00	0.001 ± 0.00	0	0	0	0	0
13	0	0	0	0	0.134 ± 0.01	0.003 ± 0.00	0.005 ± 0.00	0	0	0	0	0	0
14	0	0	0	0	0.055 ± 0.01	0	0.028 ± 0.00	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2-4. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (mg ml^{-1}) of bovine bile

	Bile Concentrations (mg ml^{-1})							
day	0	0.05	0.20	0.40	0.80	1.60	3.20	6.40
1	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00
2	0.115 ± 0.01	0.108 ± 0.01	0.100 ± 0.02	0.097 ± 0.01	0.081 ± 0.01	0.068 ± 0.00	0.088 ± 0.01	0.082 ± 0.02
3	0.205 ± 0.04	0.225 ± 0.01	0.172 ± 0.01	0.179 ± 0.04	0.152 ± 0.04	0.089 ± 0.01	0.068 ± 0.00	0.061 ± 0.02
4	2.052 ± 0.50	2.396 ± 0.53	1.500 ± 0.11	1.533 ± 0.60	1.074 ± 0.41	0.466 ± 0.05	0.066 ± 0.01	0.057 ± 0.02
5	7.580 ± 0.37	7.875 ± 0.68	7.327 ± 0.78	6.796 ± 0.45	6.378 ± 0.27	3.893 ± 0.55	0.062 ± 0.00	0.063 ± 0.00
6	8.204 ± 0.43	7.769 ± 0.45	7.590 ± 0.96	7.220 ± 0.23	6.669 ± 0.32	5.551 ± 0.11	0	0
7	7.943 ± 0.54	7.592 ± 0.66	7.651 ± 0.81	6.829 ± 0.27	6.610 ± 0.24	5.434 ± 0.10	0	0
8	7.736 ± 0.49	7.464 ± 0.28	7.226 ± 0.74	6.738 ± 0.38	0	0	0	0
9	0	0	0	0	0	0	0	0

Table 2-5. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (%) of fish bile

	Bile Concentration (%)							
day	0	1	2	4	6	8	10	12.5
1	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00	0.005 ± 0.00
2	0.342 ± 0.07	0.208 ± 0.03	0.125 ± 0.04	0.033 ± 0.01	0.025 ± 0.00	0.009 ± 0.01	0.004 ± 0.00	0.005 ± 0.00
3	1.194 ± 0.11	0.451 ± 0.08	0.245 ± 0.03	0.066 ± 0.01	0.043 ± 0.00	0.023 ± 0.01	0.006 ± 0.01	0.0002 ± 0.00
4	6.093 ± 0.28	3.742 ± 0.35	1.924 ± 0.08	0.228 ± 0.03	0.045 ± 0.01	0.040 ± 0.00	0.0003 ± 0.00	0
5	6.416 ± 0.66	4.493 ± 0.04	3.695 ± 0.07	2.856 ± 0.22	0.129 ± 0.05	0.346 ± 0.14	0.0002 ± 0.00	0
6	6.352 ± 0.91	0	0	3.550 ± 0.12	0.626 ± 0.31	1.763 ± 1.09	0	0
7	4.425 ± 1.08	0	0	0	3.080 ± 0.62	0	0	0
8	2.667 ± 0.30	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0

Table 3-1. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (%) of DMSO

	DMSO (%)						
hour	0	0.025	0.05	0.10	0.15	0.20	0.25
0	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16
24	7.362 ± 0.32	6.728 ± 0.07	6.601 ± 0.24	6.438 ± 0.16	6.291 ± 0.07	6.854 ± 0.41	6.872 ± 0.14
48	7.595 ± 0.32	7.107 ± 0.18	6.970 ± 0.17	6.563 ± 0.15	6.807 ± 0.07	6.679 ± 0.16	6.882 ± 0.10
72	6.995 ± 0.34	6.674 ± 0.04	6.448 ± 0.19	6.253 ± 0.17	6.379 ± 0.06	6.233 ± 0.05	6.305 ± 0.16
96	6.756 ± 0.40	5.952 ± 0.14	5.929 ± 0.21	5.916 ± 0.33	5.931 ± 0.10	6.063 ± 0.13	6.185 ± 0.19

Table 3-2. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (μg ml^{-1}) of pyrimethamine

	Pyrimethamine (μg ml^{-1})						
hour	control	1.0	2.0	4.0	6.0	8.0	10.0
0	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16
24	7.362 ± 0.32	6.637 ± 0.40	6.740 ± 0.29	6.640 ± 0.16	6.249 ± 0.08	6.259 ± 0.09	6.223 ± 0.12
48	7.595 ± 0.32	6.838 ± 0.41	6.805 ± 0.16	6.656 ± 0.25	6.410 ± 0.12	6.419 ± 0.14	6.285 ± 0.08
72	6.995 ± 0.34	6.611 ± 0.27	6.431 ± 0.32	6.176 ± 0.18	6.210 ± 0.09	6.118 ± 0.16	5.999 ± 0.11
96	6.756 ± 0.40	6.110 ± 0.31	6.306 ± 0.57	5.886 ± 0.30	5.685 ± 0.11	5.691 ± 0.07	5.486 ± 0.17

Table 3-3. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (μg ml^{-1}) of dimetridazole

	Dimetridazole (μg ml^{-1})						
hour	control	1.0	2.0	4.0	6.0	8.0	10.0
0	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16
24	7.362 ± 0.32	4.772 ± 0.04	3.617 ± 0.06	3.031 ± 0.12	2.975 ± 0.07	2.904 ± 0.04	2.735 ± 0.05
48	7.595 ± 0.32	4.766 ± 0.15	3.290 ± 0.09	2.526 ± 0.04	2.226 ± 0.07	2.468 ± 0.08	2.260 ± 0.04
72	6.995 ± 0.34	4.569 ± 0.13	2.975 ± 0.06	2.011 ± 0.09	1.612 ± 0.08	2.004 ± 0.10	2.158 ± 0.06
96	6.756 ± 0.40	4.351 ± 0.17	2.844 ± 0.07	1.753 ± 0.06	1.448 ± 0.03	1.864 ± 0.03	1.991 ± 0.08

Table 3-4. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (µg ml^{-1}) of metronidazole

	Metronidazole (µg ml^{-1})						
hour	control	1.0	2.0	4.0	6.0	8.0	10.0
0	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16	5.442 ± 0.16
24	7.362 ± 0.32	5.465 ± 0.34	4.487 ± 0.09	3.636 ± 0.21	3.196 ± 0.12	3.390 ± 0.02	3.186 ± 0.18
48	7.595 ± 0.32	5.335 ± 0.33	4.171 ± 0.08	2.856 ± 0.09	2.637 ± 0.05	2.709 ± 0.04	2.494 ± 0.05
72	6.995 ± 0.34	5.229 ± 0.11	4.097 ± 0.18	2.671 ± 0.12	2.501 ± 0.35	2.283 ± 0.19	2.232 ± 0.04
96	6.756 ± 0.40	4.957 ± 0.10	3.766 ± 0.17	2.539 ± 0.16	1.958 ± 0.11	2.069 ± 0.03	1.970 ± 0.05

Table 3-5. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (μg ml^{-1}) of albendazole

	Albendazole (μg ml^{-1})							
hour	control	0.1	0.5	1.0	1.5	2.0	2.5	3.0
0	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15
24	8.658 ± 0.26	8.107 ± 0.53	6.406 ± 0.52	4.259 ± 0.04	4.005 ± 0.04	4.200 ± 0.15	4.157 ± 0.25	4.057 ± 0.14
48	8.004 ± 0.44	7.478 ± 0.54	5.733 ± 0.41	3.945 ± 0.10	3.856 ± 0.05	3.943 ± 0.05	3.779 ± 0.16	3.768 ± 0.07
72	7.624 ± 0.31	7.428 ± 0.41	5.401 ± 0.15	3.721 ± 0.08	3.582 ± 0.16	3.483 ± 0.08	3.434 ± 0.19	3.539 ± 0.06
96	7.296 ± 0.57	7.099 ± 0.66	5.246 ± 0.27	3.690 ± 0.02	3.554 ± 0.16	3.341 ± 0.08	3.300 ± 0.08	3.273 ± 0.16

Table 3-6. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (μg ml^{-1}) of fenbendazole

	Fenbendazole (μg ml^{-1})							
hour	control	0.1	0.5	1.0	1.5	2.0	2.5	3.0
0	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15
24	8.658 ± 0.26	7.981 ± 0.43	5.889 ± 0.33	4.025 ± 0.12	3.902 ± 0.07	3.920 ± 0.08	3.931 ± 0.01	4.037 ± 0.09
48	8.004 ± 0.44	7.983 ± 0.58	5.783 ± 0.15	3.943 ± 0.07	3.819 ± 0.17	3.918 ± 0.10	3.651 ± 0.07	3.856 ± 0.08
72	7.624 ± 0.31	7.519 ± 0.07	5.745 ± 0.18	3.928 ± 0.11	3.752 ± 0.23	3.881 ± 0.29	3.701 ± 0.11	3.867 ± 0.22
96	7.296 ± 0.57	7.335 ± 0.44	5.505 ± 0.16	3.896 ± 0.12	3.740 ± 0.15	3.747 ± 0.18	3.609 ± 0.16	3.557 ± 0.17

Table 3-7. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (μg ml^{-1}) of mebendazole

	Mebendazole (μg ml^{-1})							
hour	control	0.1	0.5	1.0	1.5	2.0	2.5	3.0
0	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15	4.228 ± 0.15
24	8.658 ± 0.26	6.480 ± 0.20	4.108 ± 0.07	3.820 ± 0.11	3.819 ± 0.11	3.973 ± 0.14	3.911 ± 0.16	3.890 ± 0.09
48	8.004 ± 0.44	6.544 ± 0.05	3.772 ± 0.04	3.038 ± 0.16	3.055 ± 0.14	2.854 ± 0.18	3.047 ± 0.11	3.162 ± 0.26
72	7.624 ± 0.31	5.950 ± 0.34	3.326 ± 0.07	2.291 ± 0.11	2.232 ± 0.05	2.078 ± 0.17	2.181 ± 0.09	2.256 ± 0.21
96	7.296 ± 0.57	5.823 ± 0.32	2.970 ± 0.05	1.954 ± 0.06	1.987 ± 0.08	2.036 ± 0.15	2.151 ± 0.08	2.113 ± 0.15

Table 3-8. Average cell number ± s.d. of *S. vortens* (million cells ml^{-1}) cultivated in different concentrations (mg ml^{-1}) of magnesium sulfate ($MgSO_4$)

	$MgSO_4$ (mg ml^{-1})											
hour	0	5	10	20	30	40	50	60	70	80	90	100
0	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00	3.780 ± 0.00
24	9.890 ± 0.94	8.243 ± 0.30	7.930 ± 0.60	6.860 ± 0.33	6.357 ± 0.26	5.407 ± 0.14	4.743 ± 0.28	4.163 ± 0.22	3.317 ± 0.16	3.003 ± 0.04	2.873 ± 0.09	2.997 ± 0.05
48	10.130 ± 1.17	8.200 ± 0.13	8.013 ± 0.41	6.787 ± 0.38	6.277 ± 0.17	5.370 ± 0.11	4.500 ± 0.28	4.030 ± 0.16	3.233 ± 0.03	2.870 ± 0.05	2.713 ± 0.10	2.857 ± 0.05
72	10.300 ± 0.99	8.713 ± 0.06	8.093 ± 0.78	7.247 ± 0.15	6.523 ± 0.25	5.733 ± 0.58	4.530 ± 0.17	3.867 ± 0.10	3.200 ± 0.02	2.727 ± 0.08	2.667 ± 0.07	2.767 ± 0.06
96	9.610 ± 0.98	7.850 ± 0.53	7.660 ± 0.28	6.550 ± 0.07	5.850 ± 0.27	5.113 ± 0.08	4.517 ± 0.07	3.787 ± 0.04	2.983 ± 0.15	2.640 ± 0.02	2.530 ± 0.07	2.540 ± 0.04
120	8.570 ± 1.20	7.317 ± 0.42	6.993 ± 0.13	5.863 ± 0.41	5.400 ± 0.15	4.820 ± 0.09	4.110 ± 0.17	3.473 ± 0.09	2.813 ± 0.13	2.520 ± 0.16	2.247 ± 0.15	2.333 ± 0.05

Table 4.1. Pathological changes in affected tissues of IP *S. vortens*-infected angelfish

organ/location	pathological changes	tissue reactions
gastrointestinal tract	mild to moderate multifocal enteritis	congestion, edema, infiltration of eosinophilic granular cells, lymphocytes, and a moderate number of neutrophils
abdominal cavity	peritonitis	fibroblast proliferation within the mesentery, infiltration of macrophages, lymphocytes, plasma cells, and a moderate number of neutrophils
spleen	telangiectasis, fibrosis	infiltration of eosinophilic granular cells, mild fibrosis at the perimeter of melano-macrophage centers
hepatopancreas	fat and glycogen depletion	decreased hepatocyte vacuolization, moderate infiltration of mononuclear cells
kidney	nephrocalcinosis, glomerulosclerosis, granuloma formation	extensive mineral deposition, synechia, fibroblast proliferation, renal tubule: degeneration, necrosis, reactive hyperplasis/hypertrophy
ovary	granulomatous inflammation	haemorrhage, individual cell necrosis, ruptured follicles, infiltration of macrophages, lymphocytes, plasma cells, and a moderate number of neutrophils

Table 5-1. Percentage ± s.e. of macrophages producing hydrogen peroxide (H_2O_2) in angelfish inoculated with different dosages and routes of *S. vortens*

day	control (PO)	control (IP)	PO1 (5×10^6)	PO2 (1×10^7)	IP (2×10^6)
7	7.27 ± 2.14	1.77 ± 0.93	7.30 ± 2.14	14.41 ± 4.19	9.24 ± 4.22
14	3.60 ± 1.26	2.03 ± 0.19	2.22 ± 0.51	10.10 ± 4.94	4.43 ± 1.32
21	3.03 ± 1.16	6.63 ± 1.03	4.63 ± 0.89	6.30 ± 1.70	6.33 ± 1.96
28	10.27 ± 0.85	3.07 ± 2.42	20.28 ± 5.82	6.70 ± 1.56	13.64 ± 8.77

Table 5-2. Back-transformed percentage of macrophages expressing phagocytic activity in angelfish inoculated with different dosages and routes of *S. vortens*

day	control (PO)	control (IP)	PO1 (5×10^6)	PO2 (1×10^7)	IP (2×10^6)
7	2.26	1.52	1.60	2.40	1.51
14	0.59	1.03	0.85	0.86	0.90
21	0.16	0.17	0.12	0.15	0.10
28	0.14	0.10	0.14	0.13	0.13

APPENDIX A: EXPERIMENTAL PROTOCOLS

CRYOPRESERVATION OF *S. VORTENS*

Procedures

1. Harvest *S. vortens* from culture which is at or near peak density by centrifugation at 360 x g for 5 minutes.
2. Wash cells (*S. vortens*) twice in fresh culture medium (TYI-S-33), centrifuge at 360 x g for 5 min and resuspend in fresh TYI-S-33 medium.
3. Count the number of *S. vortens* by automatic cell counter (CASY®1, model TTC, Schärfe System GmbH, Germany).
4. Adjust concentration of cells to 2 x 20^7 cells ml^{-1} in fresh medium (TYI-S-33).
5. Prepare a 20% (v/v) solution of sterile DMSO in fresh TYI-S-33 medium. The solution is allowed to return to room temperature prior to use.
6. Mix the cell preparation and the DMSO in equal portions (1:1). The final concentration of the cell suspension is 1 x 10^7 cells ml^{-1} in 10% (v/v) DMSO.
7. Add antibiotics, penicillin (2,000 U ml^{-1}) and gentamicin (50 µg ml^{-1}) into the suspension.
8. Dispense 0.5 ml aliquots to 2.0 ml sterile screw-capped vials.
9. Place the vials in a refrigerator at 4°C for 10 min, and then at 0°C for another 10 minutes.
10. Place the vials in a –80°C freezer and store longterm.
11. To establish the organisms from the frozen state, immerse the vial in a water bath at 35°C for 2 minutes.
12. Immediately after thawing, aseptically transfer the suspension into 13.0 ml of fresh TYI-S-33 culture medium and incubate at 25°C.

MACROPHAGE FUNCTION TEST

I. Chemiluminescence Assay and Flow Cytometric Analysis

A. Collection of kidneys and preparation of cell suspensions

Procedures

1. Sacrifice fish by exposing to an overdose of tricane methanesulfonate (MS-222, Sigma Chemical Co., St. Louis, MO).
2. Cut along the abdominal wall of the fish to reveal all internal organs.
3. Carefully remove the kidney attached at the dorsal abdominal cavity and remove all fascia attached to the kidney.
4. Immediately place the kidney in a tissue culture plate containing 2 ml RPMI-1640 medium (Mediatech Inc., Herndon, VA).
5. Grasp a 60-mesh stainless steel sieve screen with a pair of forceps.
6. Immerse the sieve screen in the RPMI media.
7. Gently grasp the kidney with fine forceps and place it against the sieve.
8. Carefully dissociate the cells of the kidney by gently abrading the organ against the sieve with moderate pressure.
9. Transfer the cell suspension into a sterile 12 x 75 mm culture tube.

B. Washing and quantification of cell suspension

Procedures

1. Place the culture tubes containing the cell suspension in a centrifuge.
2. Centrifuge the cell suspensions for 7 min at 360 x g at room temperature (23°C).
3. Gently drain the supernatant and wash the cells by adding 2 ml PBS, pH 7.4 into each tube.
4. Using a 1000 µl automatic pipette, gently aspirate and expel the PBS several times so that the pellet is completely broken up.
5. Centrifuge the tubes, drain the supernatant and resuspend the cells in 2 ml PBS.
6. Repeat the washing steps 2 times and resuspend the cells in 1 ml PBS.
7. Mix the cell suspension using 1000 µl automatic pipette.
8. Using a 10 µl automatic pipette, add 5 µl cell suspension into a tube containing 10 ml PBS.
9. Mix the cell suspension again using a 1000 µl automatic pipette.
10. Place the tube in the automatic cell counter (CASY®1; model TTC, Schärfe System GmbH, Germany) to measure the number of cells.
11. Select the desired population of cells having size larger than 6 µm by gauging on the display chart.
12. Standardize the cell suspension to 1.0×10^6 cells ml^{-1} by adding the appropriate volume of PBS, pH 7.4.

C: Hydrogen peroxide (H_2O_2) detection

Procedures

1. Using a 1000 µl automatic pipette, add 250 µl cell suspension (0.25×10^6 cells) of the experimental and control samples to clean 12 x 75 mm tubes.
2. Add 5 µl dichlorofluorescin diacetate (DCF-DA, 5 mM) into each 12 x 75 mm culture tube.
3. Incubate the cell suspension with DCF-DA for 15 min at room temperature (23°C).
4. Add 10 µl phorbol-12 myristate 13-acetate (PMA) into each culture tube.
5. Incubate the cell suspension with PMA for 30 min at room temperature (23°C).
6. At the end of the incubation period, immediately place the cells on ice and evaluate by flow cytometry.

II. Phagocytosis Assay and Flow Cytometric Analysis

A. Preparation (sonication) of fluoresbrite microspheres

Procedures

1. Dilute 300 µl of the carboxylate-modified microspheres (FluoSpheres®, Molecular Probes Inc., Eugene, OR, 2.0 µm, No. F-8827) with 700 µl of RPMI media.
2. Centrifuge the microspheres in the RPMI for 10 minutes at 360 x g at room temperature (23°C).
3. Carefully remove the tube from the centrifuge and gently drain the supernatant, leaving the pellet in the bottom of the tube.
4. Resuspend the pellet with 2 ml RPMI using a 1000 µl automatic pipette.

5. Centrifuge, drain and resuspend the microspheres in 2 ml RPMI.
6. Repeat washing steps another 2 times, and resuspend the microspheres in 2 ml RPMI.
7. To prevent overheating of the solution during sonication, place the tube of microspheres in RPMI upright in the beaker of ice.
8. Sonicate the microspheres using the ultrasonic cell disrupter at 35% capacity for 30 sec. with 15 sec on, 15 sec off and 15 sec on.

B. Incubation of cell suspension with microspheres

Procedures

1. Obtain the cell suspension remaining from the H_2O_2 testing.
2. Centrifuge the cell suspension at 360 x g for 7 min and resuspend the cells in 1 ml RPMI supplemented with antibiotics (penicillin-streptomycin, 1000 IU ml-1 and 1000 µg ml-1, Mediatech Inc., Herndon, VA, No. MT30001CI), 10% FBS (Atlanta Biologicals, Norcross, GA, No. S11510, which has been boiled at 56°C for 30 min), and non-essential amino acid (Mediatech Inc., Herndon, VA, No. 25-025-CI).
3. Count the cell number and standardize the concentration of cells to 1×10^6 cells ml^{-1} as previously described.
4. Add 500 µl of the cell suspension (0.5×10^6 cells) into 12 x 75 mm culture tubes.
5. Add 45 µl of microspheres to all tubes to give an initial ratio of 100 beads $cell^{-1}$.
6. Add enough RPMI to each tube to give a final volume of 4.0 ml $tube^{-1}$.
7. Loosely the tube's cap and place in an incubator at 25°C, with 5% CO_2 for 18 hours.
8. At the end of an incubation period, resuspend the cells in 0.5 ml RPMI and immediately evaluate the suspensions by flow cytometry.

APPENDIX B: EQUIPMENT USED

I. *In vitro* studies on optimal requirements for the growth of *Spironucleus vortens*, an intestinal parasite of the freshwater angelfish

- sterile 4-ml screw capped glass tubes
- 16 x 125 mm sterile screw-capped glass tubes
- a compound microscope
- hemacytometer counting chamber
- weighing balance
- test tube racks
- sterile pasture pipettes
- automatic pipettes (10, 100, 1000 µl)
- glass slides & cover glasses
- 250-ml sterile tissue culture flasks
- pH meter (Accumet® pH meter 10, Fisher Scientific, Pittsburgh, PA)
- laminar flow hood
- thermostat-regulated incubators (5 units: 25°, 28°, 31°, 34° and 37°C)
- automatic cell counter (CASY®1; model TTC, Schärfe System GmbH, Germany)

II. Efficacy of various chemotherapeutic agents on the growth of *Spironucleus vortens*, an intestinal parasite of the freshwater angelfish

- 4-ml sterile screw capped glass tubes
- 16 x 125 mm sterile screw-capped glass tubes
- 250-ml sterile tissue culture flasks

- weighing balance
- a compound microscope
- test tube racks
- hemacytometer counting chamber
- sterile pasture pipettes
- automatic pipettes (10, 100, 1000 µl)
- thermostat-regulated incubator (25°C)
- automatic cell counter (CASY®1; model TTC, Schärfe System GmbH, Germany).
- glass slides & cover glasses

III. Intestinal pathogenesis of *Spironucleus vortens* infection in angelfish

1. Fish

- 20-gallon glass aquariums
- biofilter boxes with gravel and filter floss
- fish net

2. Inoculation

- automatic pipette (100 µl)
- micropipette tips
- fish net
- 12 x 75 mm sterile test tubes

3. Clinical observation

- book for records

4. Gross pathological examination

- glass slides

- fish net
- postmortem equipment (scissors, forceps)
- dissection trays

5. Light microscopic examination
    - a compound microscope
    - histopath equipment (for tissue processing)
    - glass slides and cover glasses
6. TEM & SEM examination
    - TEM (JEOL USA, Inc., Peabody, MA)
    - SEM (Philips Electron Optics, Inc., Hillsboro, OR)
    - SPI module sputter coater (SPI Supplies Division of Structure Probe, Inc., West Chester, PA)
    - Ladd critical point dryer (Ladd Research Industries, Inc., Burlington, VT)
    - postmortem equipment for tissue
    - standard TEM and SEM processing supplies

IV. Immune response of angelfish infected with *Spironucleus vortens*

1. Serum collection
    - 1.0 ml insulin syringes
    - 28-gauge needles
    - test tubes & test tube racks
    - pasteur pipettes
    - centrifuge
    - -80°C freezer

- pediatric centrifuge tubes (Microtainer, Becton Dickinson and Co., Rutherford, NJ)

2. *Spironucleus vortens* antigen preparation

   - test tubes and test tube racks
   - ultrasonic cell disrupter (Sonar Dismembrator, model 300, Fisher Scientific, Pittsburgh, PA)
   - centrifuge
   - Pasteur pipettes
   - eppendorf tubes
   - -80°C freezer

3. Double-diffusion precipitation test

   - 250 ml flasks
   - hot plate
   - 50 x 70 mm glass slides
   - agar puncher
   - automatic pipette (100 µl) and pipette tips
   - humidity chamber (plastic box)
   - weighing balance
   - Gelbond Film®

4. Localized leucocyte response

   - a compound microscope

5. Collection of kidneys and preparation of cell suspensions

   - post-mortem equipment (scissors, forceps)
   - sterile culture plates

- 12 x 75 mm sterile culture tubes
- test tube racks
- 60-mesh stainless steel sieve screens
- regular and fine forceps

6. Washing and quantification of cell suspensions

   - 12 x 75 mm sterile culture tubes
   - centrifuge
   - 10 ml pipettes
   - 10 µl automatic pipette
   - 1000 µl automatic pipette
   - pipette tips
   - automatic cell counter (CASY®1; model TTC, Schärfe System GmbH, Germany)

7. Macrophage function test: H_2O_2 detection

   - 100 µl automatic pipette and pipette tips
   - 10 µl automatic pipette and pipette tips
   - 12 x 75 mm sterile culture tubes
   - Coulter Epics XL flow cytometer (Beckmann Coulter, Hialeah, FL)
   - container for ice

8. Macrophage function test: phagocytosis of microspheres:

   Preparation (sonication) of microspheres

   - 100 µl automatic pipette and pipette tips
   - 1000 µl automatic pipette and pipette tips
   - 12 x 75 mm sterile culture tube

- centrifuge
- 250 ml beaker
- ultrasonic cell disrupter (Sonar Dismembrator, model 300, Fisher Scientific, Pittsburgh, PA)

9. Incubation of cell suspensions with microspheres

- 12 x 75 mm sterile culture tubes
- 10 µl, 100 µl automatic pipette and pipette tips
- incubator (25°C)
- Coulter Epics XL flow cytometer (Beckmann Coulter, Hialeah, FL)

APPENDIX C: REAGENTS AND CHEMICALS USED

I. *In vitro* studies on optimal requirements for the growth of *Spironucleus vortens*, an intestinal parasite of the freshwater angelfish

- *S. vortens* (ATCC, No. 50386, Manassas, VA)
- TYI-S-33 medium (ATCC, No. 350-X, Manassas, VA)
- trypan blue dye (Sigma Chemical Co., St. Louis, MO)
- Protoslo® (Carolina Biological Supply Co., Burlington, NC)
- bovine bile (B8381, Sigma Chemical Co., St. Louis, MO)
- 1 N NaOH
- 1 N HCl

II. Efficacy of various chemotherapeutic agents on the growth of *Spironucleus vortens*, an intestinal parasite of the freshwater angelfish

- *S. vortens* (ATCC, No. 50386, Manassas, VA)
- TYI-S-33 medium (ATCC, No. 350-X, Manassas, VA)
- dimetridazole (Sigma Chemical Co., St. Louis, MO)
- metronidazole (Sigma Chemical Co., St. Louis, MO)
- pyrimethamine (Burroughs Wellcome Co., Research Triangle Park, NC)
- albendazole (Sigma Chemical Co., St. Louis, MO)
- mebendazole (Sigma Chemical Co., St. Louis, MO)
- fenbendazole (Sigma Chemical Co., St. Louis, MO)
- DMSO (Sigma Chemical Co., St. Louis, MO)
- Protoslo® (Carolina Biological Supply Co., Burlington, NC)

III. Intestinal pathogenesis of *Spironucleus vortens* infection in freshwater angelfish

1. Fish
   - commercial fish diet (TetraMIN®, Tetra Sales, Blacksburg VA)
2. Infection of *Spironucleus*
   - *S. vortens* suspension
   - PBS, pH 7.4
   - tricane methanesulfonate (MS-222) (Sigma Chemical Co., St. Louis, MO)
3. Gross pathological examination
   - PBS, pH 7.4
   - tricane methanesulfonate (MS-222) (Sigma Chemical Co., St. Louis, MO)
4. Light microscopic examination
   - tricane methanesulfonate (MS-222) (Sigma Chemical Co., St. Louis, MO)
   - 10% neutral buffered formalin
   - H&E stain
   - Feulgen stain
5. TEM & SEM examination
   - cold fixative (5% glutaraldehyde, 4.4% formaldehyde, 2.75% picric acid in 0.05 M sodium cacodylate buffer at pH 7.4)
   - graded ethanol (30%, 50%, 70%, 95%) and absolute ethanol
   - Maraglas® (Electron Microscope Sciences, Fort Washington, PA)

- propylene oxide
- uranyl acetate
- lead citrate

IV. Immune response of angelfish infected with *Spironucleus vortens*

1. Serum collection
2. *Spironucleus* antigen preparation
   - PBS, pH 7.4
3. Double-diffusion precipitation test
   - agar
   - NaN_3
   - PBS, pH 7.4
4. Localized leucocyte response
5. Collection of kidneys and preparation of cell suspensions
   - tricane methane sulfonate (MS-222) (Sigma Chemical Co., St. Louis, MO)
   - PBS, 7.4
6. Washing and quantification of cell suspension
   - PBS, 7.4
   - RPMI-1640 culture medium (Mediatech Inc., Herndon, VA)
7. Macrophage function test: H_2O_2 detection

   Incubation of cells with DCF-DA and stimulation of cells with PMA
   - DCF-DA (Sigma Chemical Co., St. Louis, MO)
   - PMA (Sigma Chemical Co., St. Louis, MO)

8. Macrophage function test: phagocytosis of microspheres:

Preparation (sonication) of microspheres

- RPMI-1640 culture medium (Mediatech Inc., Herndon, VA)
- carboxylate-modified microspheres (FluoSpheres®, Molecular Probes Inc., Eugene, OR, 2.0 μm, No. F-8827)

9. Incubation of cell suspensions with microspheres

- RPMI-1640 culture medium (Mediatech Inc., Herndon, VA)
- carboxylate-modified microspheres (FluoSpheres®, Molecular Probes Inc., Eugene, OR, 2.0 μm, No. F-8827)
- penicillin-streptomycin (Fisher Scientific, Pittsburgh, PA, No. MT30001CI)
- non-essential amino acid (Mediatech Inc., Herndon, VA, No. 25-025-CI)
- Fetal Bovine Serum (Atlanta Biologicals, Norcross, GA, No. S11510)

VITA

Somboon Sangmaneedet was born on December 18, 1963 in Ratchaburi, Thailand. After graduating from high school at Benjamarachudith, Ratchaburi in March 1982, he attended the College of Veterinary Medicine at Kasetsart University in Bangkok, Thailand where he graduated with a Doctor of Veterinary Medicine (D.V.M., Honors) degree in April 1988.

He worked as a veterinarian in a private company for two years, and in 1990 he worked as an instructor in the Department of Veterinary Pathology, Division of Parasitology, College of Veterinary Medicine at Khon Kaen University, Thailand. He received a scholarship from the Royal Thai Government to pursue his MS and Ph.D. program in USA. He received a Master of Science in Veterinary Medical Sciences from the College of Veterinary Medicine, The Ohio State University in December of 1996. He then enrolled in the Ph.D. program in Veterinary Medical Sciences (Aquatic Medicine) at the Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University (VPI&SU) in 1997.

PRESENTATIONS

Sangmaneedet S. and Smith SA. Temperature Dependence and Bile Requirement of *Spironucleus vortens*, an Intestinal Parasite of the Freshwater Angelfish. 24th Annual Eastern Fish Health Workshop. Royal Pavillion Resort, Atlantic Beach, NC. March 8-11, 1999.

Sangmaneedet S. and Smith SA. Temperature Dependence and Bile Requirement of *Spironucleus vortens*, an Intestinal Parasite of the Freshwater Angelfish. 15th Annual Research Symposium. Old Dominion Ballroom, Squires Student Center, Virginia Tech, Blacksburg, VA. March 29, 1999.

Sangmaneedet S. and Smith SA. Efficacy of Various Chemotherapeutic Agents on the Growth of *Spironucleus vortens*, an Intestinal Parasite of the Freshwater Angelfish. 11th Annual Research Symposium. Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA. May 26-27, 1999.

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Sangmaneedet S. and Smith SA (1999). Efficacy of Various Chemotherapeutic Agents on the Growth of *Spironucleus vortens*, an Intestinal Parasite of the Freshwater Angelfish. Dis Aquat Org, 38(1): 47-52

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