PORTALS OF ENTRY OF *AURANTIACTINOMYXON ICTALURI* AND INCREASED INFECTION WITH *AURANTIACTINOMYXON ICTALURI* AFTER IMMUNOSUPPRESSION IN CHANNEL CATFISH *ICTALURUS PUNCTATUS* (RAFINESQUE)

By

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Monoclonal and polyclonal antibodies against *Henneguya exilis* and polyclonal antibodies against *Aurantiactinomyxon ictaluri* were produced. Using polyacrylamide gel electrophoresis and Western blot analysis, the monoclonal antibodies were shown to react specifically with a 117 kD component of *H. exilis* spores. Polyclonal antibodies were adsorbed in live catfish to reduce nonspecific background staining in immunocytochemistry. In cross-reactivity studies by indirect fluorescent antibody test (IFAT), the monoclonal antibodies against *H. exilis* were only positive for *H. exilis* whether in pure form or in tissue sections. All polyclonal antibodies were positive by IFAT for nine different species of myxosporeans or actinosporeans with the exception of the polyclonal serum against *H. exilis* which was negative for the proliferative gill disease (PGD) organism in fish tissue sections. The monoclonal and polyclonal antibodies may be useful for diagnostic and

research purposes using IFAT and immunoperoxidase tests. Polyclonal serum against *A. ictaluri* was used in IFAT on tissue sections of specific-pathogen-free (SPF) fingerlings experimentally infected by exposure to *A. ictaluri* to trace the PGD organism in catfish. Results indicated that possible portals of entry were skin, buccal cavity, and stomach and that the organisms disseminated throughout the fish via the blood. Differences in the frequencies of lesions and PGD organisms in non-gill organs were found in SPF catfish injected with sonicated *H. exilis* or *A. ictaluri* or dexamethasone prior to being challenged by live *A. ictaluri*. The possibility exists that the injection of *H. exilis* antigen may have caused limited immunosuppression while *A. ictaluri* protected the fish. In fish injected with dexamethasone prior to challenge, different stages of PGD organisms were noticed and were associated with less inflammatory reaction.

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CHAPTER I

INTRODUCTION

Approximately 100,000 surface acres of water are devoted to aquaculture in Mississippi. Between 1977 and 1990 there has been a 300% increase in acreage devoted to aquaculture (MCES, 1991b). The majority of these aquaculture acres in Mississippi are allocated to channel catfish culture. With an estimated $307,000,000 pondside value (MCES, 1991b), the catfish industry has made a major financial contribution to the state of Mississippi. Along with the direct benefits from catfish sales, businesses related to catfish culture have generated an economic value estimated to be 7 times higher than that of the fish at pondside (Bellerud, 1993).

Infectious diseases can significantly impair channel catfish production since at least 50% of annual loss are associated with one or more infectious disease organisms (MacMillan, 1985). Many of these diseases (25%) are associated with parasites, protozoans or metazoans, which are persistently present in catfish in production ponds (Durborow et al., 1991).

The Myxosporea, protozoan parasites of the phylum Myxozoa, parasitize numerous fish species and cause

substantial mortalities (Lom, 1987; El-Matbouli et al., 1992). A parasite of this phylum is also present in the catfish populations, and is now known to be the causative agent of proliferative gill disease (PGD). In 1988 it was estimated that 9% of the fish disease cases submitted to the fish diagnostic laboratory at Stoneville, Mississippi (Durborow et al., 1991) and 6% submitted nationally (Gravois, 1992) were positive for PGD organisms. Currently, cases of PGD often are not reported because fish culturists can easily recognize the gross signs of PGD and infected fish are consequently not brought to diagnostic facilities for confirmation of the disease (Bellerud, 1993). *Henneguya* sp., another myxosporean frequently found in catfish populations, is associated with mortality among young cultured channel catfish, with reports of epizootics involving losses of 95% of the fingerlings in a pond (Meyer, 1969).

Myxosporea and Actinosporea, two classes of myxozoans, were first described respectively in 1881 by Bütschli and in 1980 by Noble (Marquès, 1986). Myxosporeans were recognized as parasites of fish and actinosporeans as parasites of aquatic annelids (Janiszewska, 1955; Wolf et al., 1986). Wolf and Markiw (1984) suggested that myxosporeans and actinosporeans were not separate classes in the phylum Myxozoa but were actually alternating life cycle-stages of a single organism. However, although developmental stages

have been described in their respective fish or annelid hosts (Janiszewska, 1955; Marques, 1984), complete life cycles, particularly the infectious stages, have eluded identification. Recently it has been demonstrated that an actinosporean, *Aurantiactinomyxon ictaluri* produced by the aquatic oligochaete *Dero digitata* may be responsible for PGD in channel catfish (Styer et al, 1991; Pote et al., 1992). However the isolation of several other actinosporeans in *D. digitata* collected from catfish ponds (Bellerud, 1993; Pote and Waterstrat, 1993) raises questions about their relationship to the PGD organism found in catfish and with other myxosporeans present in catfish.

Proliferative gill disease in catfish is characterized by the presence of multicellular organisms associated with inflammatory cells in the gills and other organs (MacMillan et al., 1989; Pote et al., 1992). However, the route of infection of *A. ictaluri* into the fish, the dissemination of the parasite inside the fish, and the nature and role of the fish immune response to the organisms are still unknown. Early stages of this organism (prior to 96 hours post-infection) are also not adequately described. Lastly the stage which infects *D. digitata* has not been isolated, thus the life cycle for PGD is incomplete.

In order to elucidate the life cycle stages of the PGD organism, examine the fish immune response to *A. ictaluri* and *H. exilis*, and confirm the role of the actinosporeans found in catfish ponds to PGD, this research had the following objectives:

1. Produce monoclonal and polyclonal antibodies against *A. ictaluri* and *H. exilis* and use those antibodies in immunocytochemistry to study the life cycle stages of PGD organism.

2. Study the cross-reactivity of these monoclonal and polyclonal antibodies to *H. exilis* and *A. ictaluri* against several species of actinosporeans and myxosporeans.

3. Determine the route of infection of *A. ictaluri* into the catfish.

4. Study the distribution and stages of development of the PGD organisms in catfish organs after administration of dexamethasone or parenteral injections of the myxozoans: *A. ictaluri* and *H. exilis*.

CHAPTER II

LITERATURE REVIEW

Development of actinosporeans and myxosporeans

The general life cycle of actinosporeans has three stages, with some variations depending on the species. The first stage of development consists of the initial infection of the appropriate annelid followed by the development of a pansporocyst or pansporoblast. The initial infective form is a binucleated sporozoite which divides by mitosis to produce two important groups of cells. One group develops to yield the enveloping cells forming the epispore, and the second group creates the endospore containing two mother-cells (alpha and beta) which in turn undergo mitotic divisions resulting in the pansporocyst with sixteen cells (Marquès, 1986; Janiszewska, 1955). The second stage is gametogenesis resulting in the formation of the gametes. The sixteen cells of the pansporocyst differentiate into either male or female cells which undergo meiosis to yield a pansporoblast with eight spores. The last stage comprises sporogenesis, or the formation of spores containing sporozoites (Janiszewska, 1955 and 1957). Inside the pansporocyst a multiplication of nuclei results in spores

made of outer and inner envelopes with polar capsules, invaginated filaments and valves, and a multinucleated sporoplasm. The pansporoblasts are lysed either inside the annelid or after their release from the worm, freeing spores into the water. The organisms then unfold their valvogenic cells and take on a planktonic kind of floating structure (Janiszewska, 1955; Ormieres and Frezil, 1969; Marques, 1984; Lom and Dykova, 1992).

The developmental stages of myxosporeans in fish are similar to those previously described with the actinosporeans. Most stages and structures of both groups reveal a considerable morphological resemblance. There are some variations in the spore formation and spore structure (actinosporeans spores are diploid while myxosporean spores are haploid), in the structure of pansporoblast, and the number of spores present per pansporoblast depending on the species (Lom and Dykova, 1992).

Pathology of myxosporeans in fish

The relationship between myxosporean parasites of many fish species and the actinosporean parasite counterpart in the oligochaetes is currently under investigation in laboratories worldwide. In the catfish industry in the United States, the most important myxosporean found in catfish is the causative agent of PGD. The infective stage is now known to be a triactinomyxid myxozoan, *Aurantiactinomyxon* sp. (Burtle et al., 1991; Styer et al.,

1991; Pote et al., 1992). Infection with this organism is characterized by histozoic myxozoan stages in channel catfish gills and other organs. Clinical signs associated with PGD are swollen, fragile gills with a red and white mottled appearance which resulted in the descriptive term of "Hamburger Gill Disease". The gills bleed easily when touched and are responsible for severe respiratory problems often manifested by fish lying listlessly along the pond bank even when dissolved oxygen concentrations are at sufficient levels (MCES, 1991a). Histopathology of PGD infected gills show severe congestion and inflammation leading to a granulomatous branchitis. Inflammatory cells comprise a mixed-cell population consisting of lymphocytes, neutrophils, and macrophages. Inflammatory foci are adjacent to gill filament cartilage and gill lamellae are fused due to inflammatory cell infiltrates and epithelial hypertrophy and hyperplasia (McMillan et al., 1989; Burtle et al., 1991). Deeply basophilic parasites are randomly scattered throughout thickened gill filaments. There is a direct correlation between the intensity of the lesion and the number of basophilic parasites or plasmodia per filament. In the latter stages, necrosis and degeneration of the cartilaginous skeleton of gill rays occurs resulting in focal hemorrhages. Interestingly, plasmodia are observed in other non-gill organs, specifically in the spleen, brain, vasculature of the liver, and the interstitium of the trunk

and head kidney. However, these organs rarely display inflammatory sequelae (McMillan et al., 1989). Subclinical infections also occur with less obvious signs. Recent research has shown that the parasite is ubiquitous in most catfish populations, but the significance is unknown (Bellerud, 1993).

Henneguya exilis Kudo is another myxosporean parasite shown to have a predisposition for channel catfish gill tissues (Duhamel et al., 1986; Garden, 1992). McCraren et al. (1975) found that most of the seventeen *Henneguya* spp. described from North American freshwater fishes were histozoic and that the organisms encompassed seven different manifestations of *Henneguya* spp. infections in channel catfish. Infections may occur in gills, barbels, adipose fins, subcutaneous tissues, and sclera and muscles of the eye. Two types of branchial (gill) infections are known; the first, causing high mortality mainly among young replacement stock (Duhamel et al., 1986), is the interlamellar form that develops cysts between gill lamellae. More specifically, *Henneguya* sp. spores developing among the basal cells between gill lamellae cause hyperplasia of the basal cells and hypertrophy of the gill lamellar tissue resulting in loss of respiratory function. The second, the intralamellar form, is considered less pathogenic and causes cysts within gill lamellae. Three cutaneous forms have been described, a papillomatous form

with large white and granular tumor-like lesions, a skin infection showing cysts on the external body surface, and a rare form occurring as long narrow white cysts in the tissue of the adipose fin. Also known are rare forms occurring in the connective tissue surrounding the mandibular teeth and in the gall bladder. Bowser et al. (1985) stated that *Henneguya* sp. lesions are usually focal or multifocal and should be differentiated from the more diffuse hyperplasia of gills seen in problems related to water quality such as high hydrogen sulfide or sodium cyanide levels and to dietary deficiency like the lack of pantothenic acid. Many other causes of epithelial hypertrophy and hyperplasia leading to fusion of gill lamellae have been added to that list including intoxication by heavy metals and pesticides, high nitrogenous waste levels in water, and chronic external parasitic, fungal and bacterial infections (Duhamel et al., 1986).

Epidemiology of myxozoan diseases in fish

Myxosporeans were first described in 1881 by Bütschli (Levine et al., 1980) in cold-blooded vertebrates (predominantly fish), and actinosporeans have been known since 1899 (Wolf and Markiw, 1984) in invertebrates (mostly annelid). However the living forms of both parasites outside their known hosts remain the subject of scientific debate. In particular, Wolf and Markiw (1984) stated that myxosporean spores from infected fish were not infective for

fishes and similarly actinosporeans from oligochaetes were incapable of infecting other worms. Studies on the causative agent of salmonid fish whirling disease, *Myxobolus cerebalis*, showed that spores from infected fishes were infective to an aquatic oligochaete only after several months of "aging" in mud (Hoffman and Putz, 1969; Uspenskaya, 1978; Wolf and Markiw, 1984). However, attempts to infect oligochaetes with spores from *Ceratomyxa shasta* infected fish have been unsuccessful.

The relationship of several species of myxosporeans and actinosporeans has recently been proven. It is now postulated that instead of the organisms being separate species depending on the host, species of both classes of the phylum Myxozoa are actually alternating life stages of a single organism. This was confirmed by the work of Markiw and Wolf (1983) and Wolf and Markiw (1984) indicating a two-host life cycle including a fish and an invertebrate intermediate in whirling disease in salmonid fish. This original work has been confirmed by several investigators demonstrating the conversion of *Myxobolus cerebralis* spores released into the water from an infected salmonid fish into the actinosporean *Triactinomyxon gyrosalmo* after ingestion of the *M. cerebralis* spores by a tubificid oligochaete *Tubifex tubifex* (Markiw and Wolf, 1983; Wolf and Markiw, 1984; Wolf et al., 1986; Markiw, 1986, 1989a, 1989b). Yokoyama et al. (1991) confirmed that another actinosporean,

Raabeia sp., infecting the oligochaete *Branchiura sowerbyi* is converted into *Myxobolus* sp. in goldfish.

In early investigations of PGD affecting commercial ponds of channel catfish, the etiologic agent was considered to be *Henneguya exilis* (McCraren et al., 1975; Bowser and Conroy, 1985; Haskins et al., 1985; Duhamel et al., 1986). However, often either PGD was diagnosed in catfish where *H. exilis* was not found (McMillan et al.,1989) or conversely *H. exilis* was observed in channel catfish without PGD symptoms (Kent et al., 1987). Other studies suggested that the etiologic agent of PGD might be *Sphaerospora* sp. (McMillan et al., 1989; Hedrick et al., 1990). Whatever the identity of the agent, transmission of PGD to uninfected catfish was successful only after exposure to water or mud from ponds with PGD infected catfish but not from infected catfish to uninfected catfish (Burtle et al., 1991; McMillan et al., 1989). Further studies showed the myxosporean-like parasite present in gills of catfish infected with PGD to be related to *Dero digitata* infected with *Aurantiactinomyxon* sp. (Styer et al., 1991; Burtle et al., 1991; Pote et al., 1992). Styer et al. (1991) were able to create PGD lesions in gills of catfish fry infected either with live *D. digitata* bearing *Aurantiactinomyxon* sp. or with squashes of *D. digitata* containing mature spores of *Aurantiactinomyxon* sp. Pote et al. (1992) infected fish by direct exposure to pure *Aurantiactinomyxon* sp. Although the infective organism for

PGD is now known, the infective stage for *D. digitata* has not been identified. An infective spore stage has not been isolated in the catfish similar to that observed in whirling disease of salmonid fish. McMillan et al. (1989) showed an incomplete sporogony of the stages of PGD organisms found in fish over a two-month period. This would suggest that catfish might be an aberrant host for the PGD organism.

Experimental conditions and epidemiological factors may play a role in the development of myxosporeans in fish. Kent and Hedrick (1987) hypothesized that water temperature, along with the fish's immune system may have prevented complete sporogony of the proliferative kidney disease (PKD) organism that infects rainbow trout, *Oncorhynchus mykiss*. However, in the case of PGD in channel catfish, McMillan et al., (1989) found that fish immune responses were nondetectable at most non-gill sites despite poor water quality in some catfish ponds which could enhance the possibility of parasitism. Fish age (Groff et al., 1989) and number of infective organisms per fish determine the severity of myxosporean diseases. Markiw (1992) demonstrated that the severity of *Myxobolus cerebralis* infections in rainbow trout decreased with increased age of fish and that Myxosporean spores could only be recovered from those fish infected with at least 100 *Triactinomyxon*. The number of myxosporean spores recovered from infected

fish gradually increased with an increase of *Triactinomyxon* numbers up to 10,000 where a plateau was observed.

Fish immune response to myxosporeans

Antibody production in poikilotherms and inflammatory reactions in fish are known to be temperature dependent (Finn and Nielsen, 1971). More studies are required to elucidate the nature of the immune response and the presence or absence of humoral antibodies in cases of myxosporidian infections. Dykova and Lom (1978) speculate that increased water temperatures cause activation of the immune response of perch and ruff which in turn leads to early and rapid destruction of *Henneguya* cysts in the gills. Cell-mediated immunity and non-specific cytotoxicity may be major components of fish effector defense mechanisms against parasites. The granulomatous branchitis involved in the infection of channel catfish by *Henneguya exilis* Kudo is suggestive of cell-mediated immunity (Duhamel et al., 1986). The presence of PGD organisms in non-gill organs of channel catfish without extensive host response, and absence of antibody response in *Myxosoma cerebralis* infections in rainbow trout have been interpreted as signs of immunosuppression or antigenic mimicry (McMillan et al., 1989; Dykova and Lom, 1978). In the cases of PGD organisms in channel catfish and PKD organisms in rainbow trout, the activity of the host's immune system has been suggested as the cause for incomplete sporogony.

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CHAPTER III

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST *HENNEGUYA EXILIS* KUDO

Abstract

Monoclonal (Mab) and polyclonal antibodies (Pab) were produced against *Henneguya exilis* Kudo. *H. exilis* spores were collected from cysts in gills of infected channel catfish, *Ictalurus punctatus*, sonicated, and subsequently used as antigen. Among 18 positive antibody producing hybridomas obtained, three were selected and shown by class and subclass typing to have similar immunoglobulin μ heavy and κ light chains. The three selected Mab to *H. exilis* reacted to *H. exilis* spores or *H. exilis* cysts in tissue sections using indirect fluorescent antibody, immunoperoxidase techniques, or in Western blots. These Mab did not cross-react with an unidentified myxosporean and *Aurantiactinomyxon ictaluri* spores. Polyclonal sera to *H. exilis*, used prior to absorption or adsorbed in live catfish, reacted with pure preparations of spore stages of *H.exilis*, *A. ictaluri*, and the unidentified myxosporean, but failed to recognize the proliferative gill disease (PGD) organism in formalized or frozen catfish tissue sections. This indicated the presence of stage specific antigens in

the PGD organism. Differences in the number of positive reactions by adsorbed and unadsorbed sera in Western blots suggested that the *H. exilis* organism undergoes host antigen mimicry.

Introduction

The myxosporean *Henneguya* sp. has been reported in cultured channel catfish, *Ictalurus punctatus* (Rafinesque), from the south central United States (McCraren et al., 1975; Minchew, 1977; Duhamel et al., 1986) and in other fish species elsewhere in the world (Dykova and Lom, 1978; Kalavati and Narasimhamurti, 1985). Infection with this parasite has resulted in high mortalities in young replacement stock in channel catfish operations (McCraren et al., 1975; Duhamel et al., 1986). Preliminary findings in our laboratory have indicated that this parasite is present subclinically year round in catfish collected from production ponds. The significance of these subclinical infections is unknown (Chenney et al., 1994).

Depending on the species, *Henneguya* infects various fish tissues including gills, barbels, adipose fins, subcutaneous tissue, skin, connective tissue of the head, sclera, muscle of the eye, and gall bladder (McCraren et al., 1975; Minchew, 1977; Dykova and Lom, 1978; Duhamel et al., 1986). *Henneguya exilis* Kudo has a predisposition for gill tissues, however the similarity of tissue stages of this organism to the multinuclear trophozoite stages of

other myxosporeans complicates the identification of this parasite (Bartholomew et al., 1989a). In early reports of proliferative gill disease (PGD) in channel catfish, it was thought that *Henneguya* sp. was the causative organism (Bowser and Conroy, 1985; Thune, 1991) since the tissue cyst stages of this organism and the myxozoan stage of PGD were often found together in the gills and looked similar in morphology. However, recent evidence indicates that the *H. exilis* and the myxozoan species causing PGD (*A. ictaluri*) are not one and the same (Styer, Harrison and Burtle, 1991; Pote et al., 1992).

The complete life cycle of *Henneguya* sp., like those of most myxosporeans, is unknown. *Henneguya* sp. tissue cysts and spore stages have been described presently, however the stage which infects fish, the initial site of infection of this early stage, and the subsequent distribution of the organism in body tissues have not been determined. Alternate life stages in an invertebrate intermediate host have been proven for the myxosporean *Myxobolus cerebralis* causing salmonid whirling disease (Wolf and Markiw, 1984) and for the organism responsible for proliferative gill disease in channel catfish (Styer et al., 1991; Pote et al., 1992), however this has not been proven with *Henneguya* sp.

Serological techniques are useful in research and diagnosis for identifying different species or life stages

of parasites. Recently investigators have used monoclonal antibodies to characterize the myxosporean *Ceratomyxa shasta* (Bartholomew et al., 1989a). The purpose of this study was to produce and characterize monoclonal and polyclonal antibodies to *Henneguya exilis* Kudo, and to use them in immunocytochemistry.

Materials and Methods

Preparation of Henneguya antigen

Henneguya exilis spores used for production of monoclonal and polyclonal antibodies (Mab and Pab) were isolated from the gills of channel catfish, *Ictalurus punctatus* (Rafinesque) obtained from a catfish processing plant in Macon, Mississippi or catfish production ponds in the Mississippi Delta. Catfish were necropsied, *H. exilis* cysts were identified microscopically, excised from the gills and placed in sterile water. Cysts were washed with three exchanges of sterile water. The cysts were penetrated with a sterile 27 G needle and the spores were aspirated into a sterile syringe. Spores collected from the cysts were resuspended in sterile water, and centrifuged at 500 x g at 10°C for 10 min. The supernatant was decanted, the pellet was resuspended in sterile water and centrifuged again at 500 x g at 10°C for 10 min. This procedure was repeated three times. Spore were enumerated using a hemacytometer (Fisher Scientific, Norcross, GA) and adjusted

to 5 x 10^6 spores/ml in sterile water. These spores were placed on ice and sonicated using a cell disruptor (Branson Sonifier Cell Disruptor, Danbury, CT) at maximum power (61 watts) four pulses at 20 s each. The solution of lysed *H. exilis* spores was divided into aliquots of 0.5 ml and kept at -20°C until use.

Production of monoclonal antibodies

RBF/Dn mice (Jackson Laboratories, Bar Harbour, ME) were primed with an intraperitoneal (IP) injection of 0.2 ml of sonicated *H. exilis* spores (1.0 x 10^6 / mouse) : 0.2 ml of Freund's complete adjuvant (Sigma, St. Louis, MO). A second IP inoculation of 0.2 ml of sonicated *H. exilis* spores (1.0 x 10^6): 0.2 ml of Freund's incomplete adjuvant (Sigma) was given three weeks after the priming inoculation. One week after the second inoculation the third, fourth, and fifth IP inoculations were given at 4 day intervals using 0.1 ml of the sonicated *H. exilis* spores (5.0 x 10^5). Two days after the third injection, blood was collected from the mice and tested to confirm the presence of specific antibodies against *H. exilis* spores using an indirect fluorescent antibody test (IFAT). Three days after the fifth injection, the spleen was removed and a single cell suspension was prepared in RPMI-1640 (Fisher, Norcross, GA) supplemented with 1% L-glutamine and 100 U penicillin-streptomycin/ml. The splenocytes were mixed with the FOX-NY myeloma cell line (Hyclone Laboratories, Logan, UT), at a

5:1 ratio (Taggart and Samloff, 1983) and allowed to fuse in the presence of 50% polyethylene glycol-4000 (Gefter et al., 1977). To select for the myeloma-lymphocyte hybrids, AAT medium (final concentration 7.5 x 10^5 M adenine, 8.0 x 10^7 M aminopterin, 1.6 x 10^5 M thymidine) (Taggart and Samloff, 1983) made in RPMI supplemented with 10% fetal bovine serum, 1% L-glutamine and 10 U penicillin-streptomycin/ml was added to the cells as they were dispensed into the wells of microtitration plates at a volume of 0.2 ml/well (4 x 10^5 spleen cells) (Ainsworth et al., 1990). Hybridomas were assayed for specific antibody production to *Henneguya* sp. spores using IFAT. Those hybridomas producing antibodies against *Henneguya* sp. spores were cloned by limiting dilution. The supernatant from wells with single clones showing sufficient growth was tested for antibody using IFAT. Those clones which tested positive for *Henneguya* sp. spores were expanded in tissue culture flasks containing RPMI with 10% fetal bovine serum, 1% L-glutamine, and 100 U penicillin-streptomycin/ml. Cells were overgrown at 37°C until dead and the tissue culture fluid was harvested and stored at -20°C.

Immunoglobulin class and subclass typing

The immunoglobulin isotypes and subclasses of the Mab were determined by an enzyme-linked immunosorbent assay (ELISA) using alkaline phosphatase labeled goat anti-mouse immunoglobulin subclass specific antibodies (Southern

Biotechnology Associate Inc., Birmingham, AL). Briefly, wells of a polyvinylchloride plate (Fisher Scientific, Norcross, GA) were coated with 50 μl of 1% poly L-lysine solution in PBS per well and incubated overnight at 4°C. The plates were rinsed after 24 h and sonicated *H. exilis* spores (5 x 10^6 spores/ml) in carbonate buffer were added (50 μl per wells) and the plates were incubated overnight at 4°C. The wells were washed three times with PBS and incubated for one hour with borate-buffered saline containing 0.25% bovine serum albumin (BSA) and 0.05% Tween 20. The wells were washed, hybridoma supernatants were added (50 μl per well) and incubated overnight. The plate was washed with PBS and incubated with the alkaline phosphatase labeled immunoglobulin subclass specific antibodies for 4 h. After three washes with PBS, p-nitrophenyl phosphate disodium (Sigma) in diethanolamine buffer ph 9.8 was added. The resulting colorometric changes were measured after 30 min using a Titer-Tek Multiskan plate reader (Flow Laboratories, McLean, VA) at 405 nm (Waterstrat et al., 1989).

Production of polyclonal antibodies

Sonicated *H. exilis* spores (1 x 10^6) in 0.75 ml were mixed with Freund's complete adjuvant in 1:1 ratio and injected into a New Zealand white rabbit (Myrtle's Rabbitry, Thompson Station, TN) as two 0.5 ml subcutaneous injections behind each scapulum and 0.5 ml intramuscularly. The second

injection of sonicated *H. exilis* spores (1 x 10^6) mixed with Freund's incomplete adjuvant in 1:1 ratio was administered four weeks later in an identical manner as the first injection. The third injection was given as sonicated *H. exilis* spores (1 x 10^6) five weeks after the first injection. Five days after the third inoculation, the rabbit was injected with an analgesic (acepromazine) then bled from the marginal ear vein, and the antiserum was tested for specificity against *H. exilis* spores using IFAT. Part of the rabbit serum was also adsorbed by injecting the serum into the caudal vein and into the peritoneal cavity of specific pathogen free (SPF) channel catfish (from the Aquatic Medicine Laboratory of the College of Veterinary Medicine at Mississippi State University) at the rate of 1.0 ml/400 gm of catfish weight. The fish was bled 4 h and 24 h after injection of the rabbit serum (Jensen and Hedrick, 1987).

Specificity of antibodies was tested by cross-reacting Mab and Pab produced against *H. exilis* to an unidentified myxosporean isolated from the skin of bluegill fish (*Lepomis macrochirus*) and to *Aurantiactinomyxon ictaluri* spores.

Indirect fluorescent antibody test

Monoclonal and Pab (1/20) were incubated for 30 min at 37°C with myxozoan spores air-dried onto glass slides or tissue sections containing PGD and *Henneguya* sp. tissue stages placed on clean glass microscope slides. All

specimens were fixed in 100% cold acetone for 15 min. Tissue sections analyzed were from fresh frozen tissues or previously formalin fixed tissues embedded in paraffin. Embedded tissue sections were deparaffinized, rehydrated, and rinsed in distilled water prior to use. Specific antibodies were detected using goat fluorescein isothiocyanate (FITC)-conjugated anti-mouse or anti-rabbit immunoglobulins (Sigma, St. Louis, MO). Washes were done with phosphate buffered saline (0.01 M phosphate and 0.4 M sodium chloride PBS) pH 8.0. Coverslips were mounted on slides using Vectashield mounting media (Vector Laboratories, Burlingame, CA). Spores and tissue sections were examined microscopically using a Zeiss standard microscope (Carl Zeiss, Oberkochen, West Germany) with an epi-fluorescence condenser and a 100 W xenon arc lamp. All immunofluorescence tests included a negative control using negative hybridoma fluid and a positive control using sera from the mice immunized for the production of hybridomas.

Immunoperoxidase test

Pure spores and infected tissue sections were first processed as outlined previously except deparaffinized and rehydrated tissue sections were not fixed in cold acetone. After quenching endogenous peroxidase activity with 0.3% (v/v) hydrogen peroxide in methanol for 30 min, slides were incubated overnight at 4°C in a refrigerator with monoclonal and polyclonal antibodies. Washes were done with PBS pH 7.4

(0.01 M phosphate and 0.14 M sodium chloride). Presence of specific antibodies for *H. exilis* spores was detected using Vectastain ABC kit (Vector Laboratories) containing avidin and biotinylated horseradish peroxidase reagents. Biotinylated anti-mouse or anti-rabbit immunoglobulins were used for Mab and Pab, respectively. Sections were counterstained with haematoxylin. Slides were coverslipped with permount and observed using routine light microscopy.

Transmission electron microscopy

Gills with *H.exilis* cysts were excised and maintained overnight in McDowell's fixative, washed in 0.1 M phosphate buffer Ph 7.2, and dehydrated in a graded ethanol series to 70% ethanol. The specimens were infiltrated with a mixture of LR White resin (Electron Microscopy Sciences, Fort Washington, PA) : 70% ethanol at a 1:1 ratio, followed by 100% resin for 1 h and incubated overnight in 100% resin at 22°C. Samples in resin were polymerized at 50°C overnight. Thin sections were cut with a diamond knife using an ultramicrotome (RMC MT 6000, Leica Inc., Derfield, Illinois), and collected on Formvar-coated grids. Samples were blocked for 15 min on drops containing 4% BSA diluted in Tris buffer (0.02 M Tris-Hcl Ph 7.3, 0.5% Tween 20, 0.5% Triton X-100 and 0.5 M NaCl). Grids were transferred to 10 microliter drops composed of monoclonal antibodies diluted 1:2 in Tris buffer. After 5 h incubation at 22°C in a humidity chamber, grids were washed three times for 10 min

with Tris buffer. Grids were then floated onto drops containing goat anti-mouse immunoglobulins conjugated to 5 nm gold beads (Biocell, Cardiff, UK) diluted 1:10 in Tris buffer. Sections were stained with uranyl acetate, examined and photographed using a JEOL 100cx transmission electron microscope operated at an acceleration voltage of 60 kV (Danforth et al., 1992; Miller and Brown, 1993).

SDS-PAGE and Western-blot

The SDS-PAGE was carried out according to the methods of Laemmli (1970) and those of Bio-Rad Laboratories for the Protean II system (Bio-Rad Laboratories, Hercules, CA). *Henneguya exilis* extracts were run on 10% acrylamide gels. Samples were initially electrophoresed through the stacking gel at 100 V and then raised to 200 V for the running gel. Molecular weight standards (high and low) were run on each gel and all proteins detected by Coomassie brilliant blue or silver stain. Following electrophoretic separation of lysed *H. exilis* and *A. ictaluri* spores, and lysed fish gill tissues, the proteins were transferred to nitrocellulose membrane by electroblotting. Nitrocellulose membranes were probed with Mab or Pab and then with the appropriate peroxidase-labeled goat anti-mouse or anti-rabbit immunoglobulins (Sigma) (Bartholomew et al., 1989a; Bartholomew et al., 1989b).

Results

A total of 18 hybridomas specific for *H. exilis* spores were obtained from one fusion. All 18 hybridomas reacted strongly with pure *H. exilis* spores (Fig 3.1A). Three of these hybridomas (MH16c1, c3, c10) were selected for further characterization. The isotypes of the 3 Mab were μ heavy chain and κ light chain (Table 3.1). The Mab and Pab were confirmed to be specific for *H. exilis* based on the IFAT. The Pab reacted more strongly than did Mab (Fig 3.1B). On individual spores, fluorescence was more localized on valves than in the sporoplasm or on the polar capsules. However after the polar capsules were extruded they were fluorescent.

When Mab and Pab were tested by IFAT for their reactivity to the unidentified myxosporean isolated from the skin of bluegill fish and *A. ictaluri* the Pab was observed to react with both organisms while the Mab did not react with either organism (Fig 3.1C and D). Fluorescence on *A. ictaluri* was localized in the epispore including the three appendages and the polar capsules. The sporoplasm of the actinosporean was less fluorescent. The unidentified myxosporean showed fluorescence in all parts of the organism.

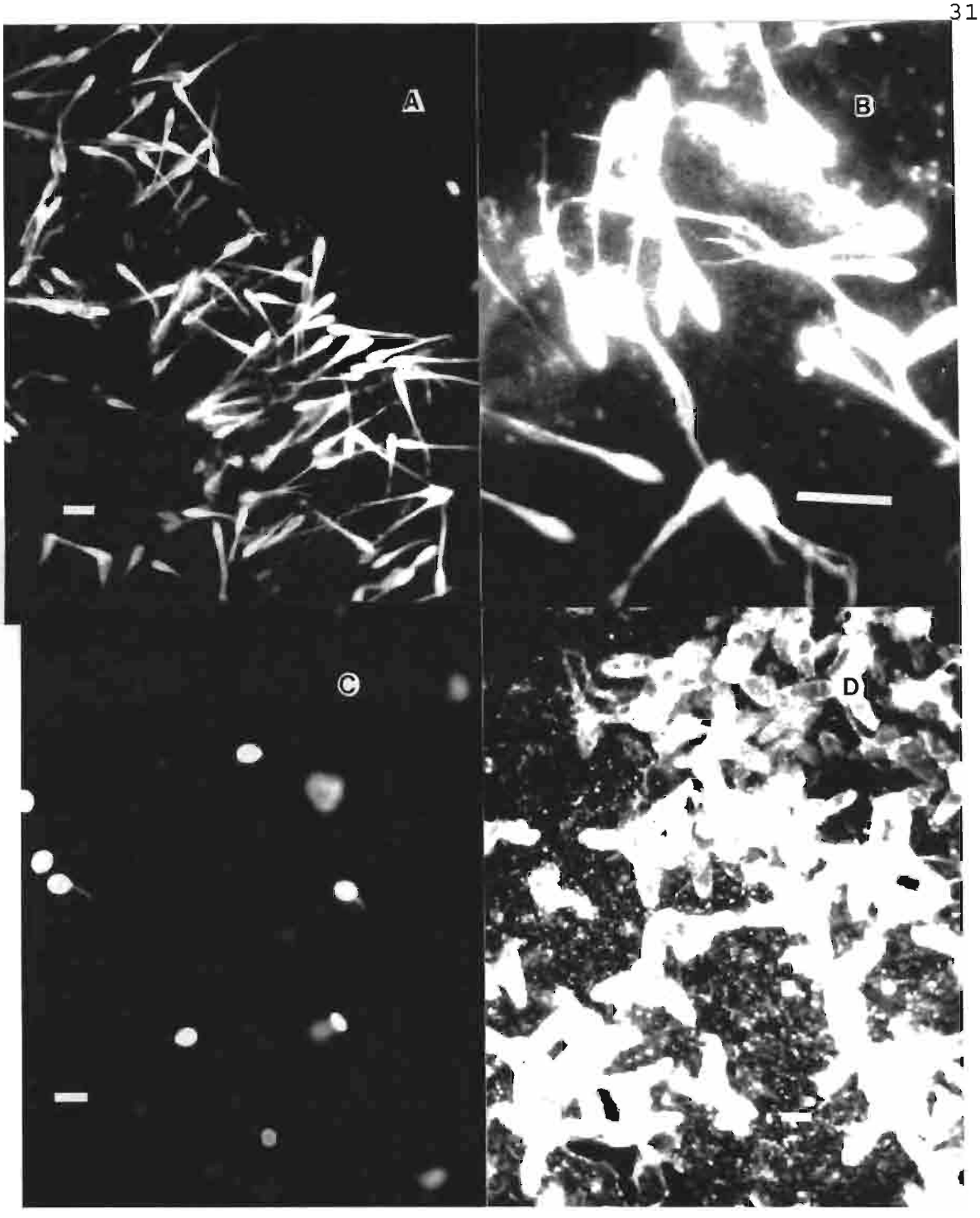

Figure 3.1. Indirect fluorescent antibody reaction of pure spore preparations. (A) *Henneguya exilis* probed with monoclonal antibodies to *H. exilis*. (B) *Henneguya exilis* probed with polyclonal serum to *H. exilis*. (C) Unidentified myxosporean isolated from bluegill fish skin nodules probed with polyclonal serum to *H. exilis*. (D) *Aurantiactinomyxon ictaluri* probed with polyclonal serum to *H. exilis*. Bars=10μm

Table 3.1. Characterization of monoclonal antibodies specific for *H. exilis* spores.

		Antigens detected by:							
		IFAT[1]				IMPX[2]			
Mab[3]	Isotype	A[4]	B[5]	C[6]	D[7]	A	B	C	D
MH16c1	IgM,k	+[8]	-[9]	+/-	+/-	+	-	+/-	+/-
MH16c3	IgM,k	+	-	+/-	+/-	+	-	+/-	+/-
MH16c10	IgM,k	+	-	+/-	+/-	+	-	+/-	+/-

[1] IFAT= Indirect fluorescent antibody test; [2]IPMX= Immunoperoxidase test; [3]Mab= Monoclonal antibody; [4]A= Pure *H.exilis* spores; [5]B= Pure unidentified myxosporean from bluegill fish and *A. ictaluri* spores; [6]C= *Henneguya* sp./ PGD infected both in frozen tissue sections; [7]D= *Henneguya* sp./ PGD infected both in formalin fixed tissue sections; [8]+= positive; [9]-= negative.

Results of SDS-PAGE of sonicated spores of *H. exilis* and *A. ictaluri* and lysed catfish gill tissues showed several bands detected by silver staining (Fig 3.2). Distinct dissimilar band patterns could be noticed among all three groups of proteins. Probing Western blots of ground fish gill tissues, *A. ictaluri* or *H. exilis* with Mab resulted in a positive reaction with a 117 kD protein of *H. exilis*. Several bands were present when the Pab were reacted in an identical fashion with ground fish gill tissues or *H. exilis* (Fig 3.3). The polyclonal serum adsorbed in live catfish reacted with fewer proteins of *H. exilis* (104, 95, 93, 88, 72, and 62 kD) than did the unadsorbed serum (140, 117, 104, 95, 93, 88, 72, 62, and 30 kD). The major antigenic determinant of *H. exilis* for both adsorbed and unadsorbed polyclonal sera was a protein having a mass of 104 kD. The unadsorbed polyclonal serum reacted to few bands (152, 140, 129, 109, 80, and 77 kD) on the ground catfish gill tissues as compared to the number of bands present on SDS-PAGE gel (Figure 3.2) while the adsorbed serum reacted to none.

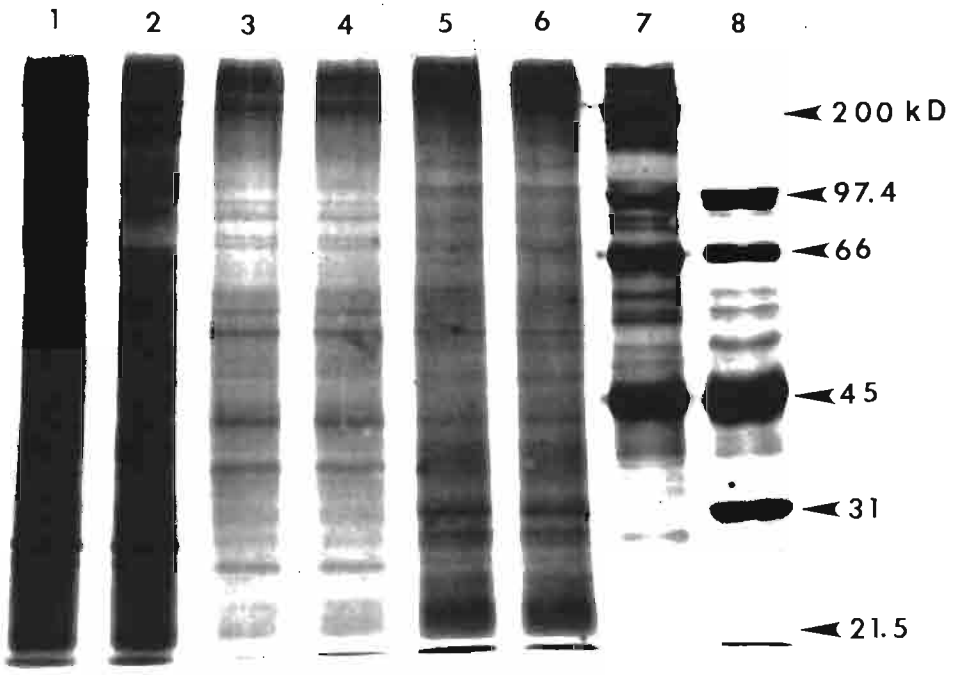

Figure 3.2. SDS-PAGE (10% acrylamide) of ground SPF catfish gill tissues (lanes 1 and 2), sonicated spores of *Aurantiactinomyxon ictaluri* (lanes 3 and 4) and *Henneguya exilis* (lanes 5 and 6). Lanes 7 and 8 are high and low molecular weight standards, respectively. Numbers on the right correspond to the molecular weight markers in kD.

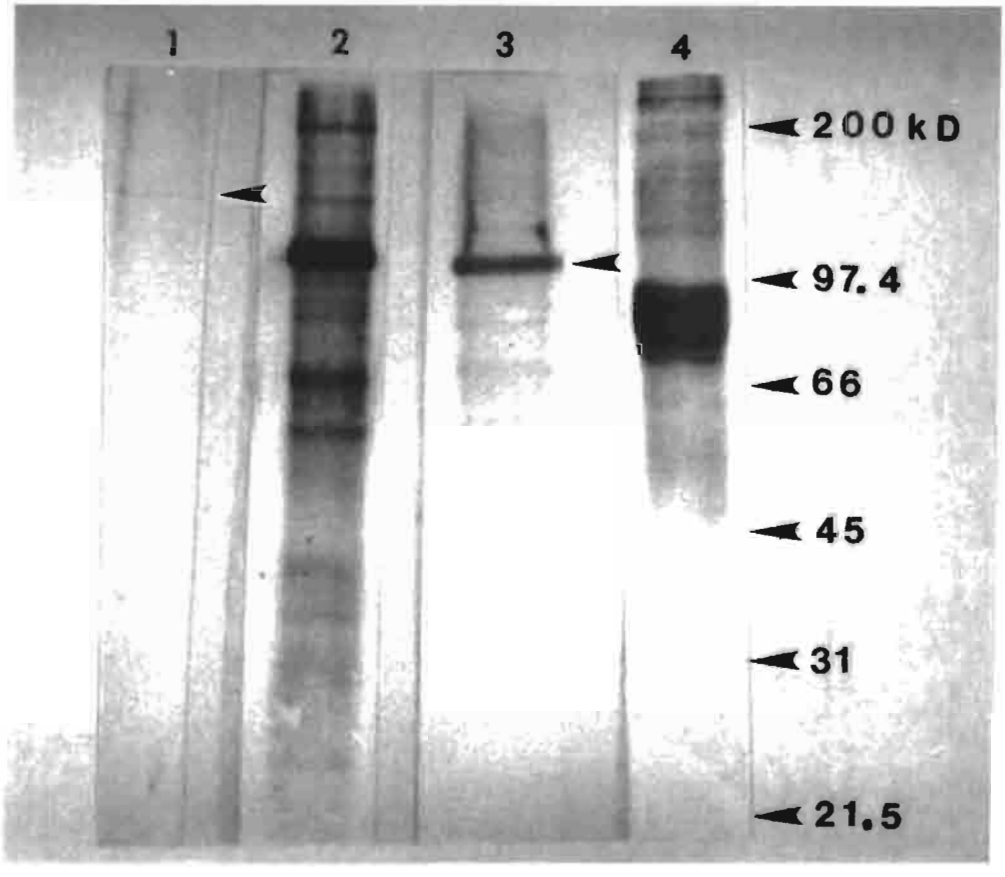

Figure 3.3. Western blot analyses of sonicated *Henneguya exilis* using monoclonal antibodies 117 kD band(lane 1), unadsorbed and adsorbed polyclonal sera 104 kD major band (lanes 2 and 3 respectively), and of ground SPF catfish gill tissues using unadsorbed polyclonal serum (lane 4). Numbers on the right correspond to the molecular weight markers in kD.

Tissue sections of *Henneguya* sp. infected catfish gills examined by IFAT and immunoperoxidase test (IMPX) using Mab MH16c1, c3, c10, and unadsorbed and adsorbed polyclonal sera to *H. exilis* were all strongly positive for the spores whether in cysts or not (Fig 3.4). However the unadsorbed polyclonal serum to *H. exilis* caused high background staining of fish tissues. In similar tests on PGD infected tissue sections, none of the antibodies cross-reacted with the PGD organisms.

No differences were observed in the amount of fluorescence observed in tissues frozen or formalin-fixed which had been deparaffinized and rehydrated (Fig 3.4A and B). However better cyst and tissue morphology, and less dissociation of tissue sections from microscope glass slides were noticed when using formalin-fixed tissues.

Electron microscopy studies indicated that the location of antigenic sites for Mab MH16c1 were primarily on the surface of the valve walls of *H. exilis* spores (Fig 3.5A). No suture defining valves was present on the tails but similar reaction was observed on the surface (Figure 3.5B). The Mab also appeared to react to material surrounding *H. exilis* spores within tissue cysts but not on the fish tissues. No reaction was detected on uninfected fish tissues.

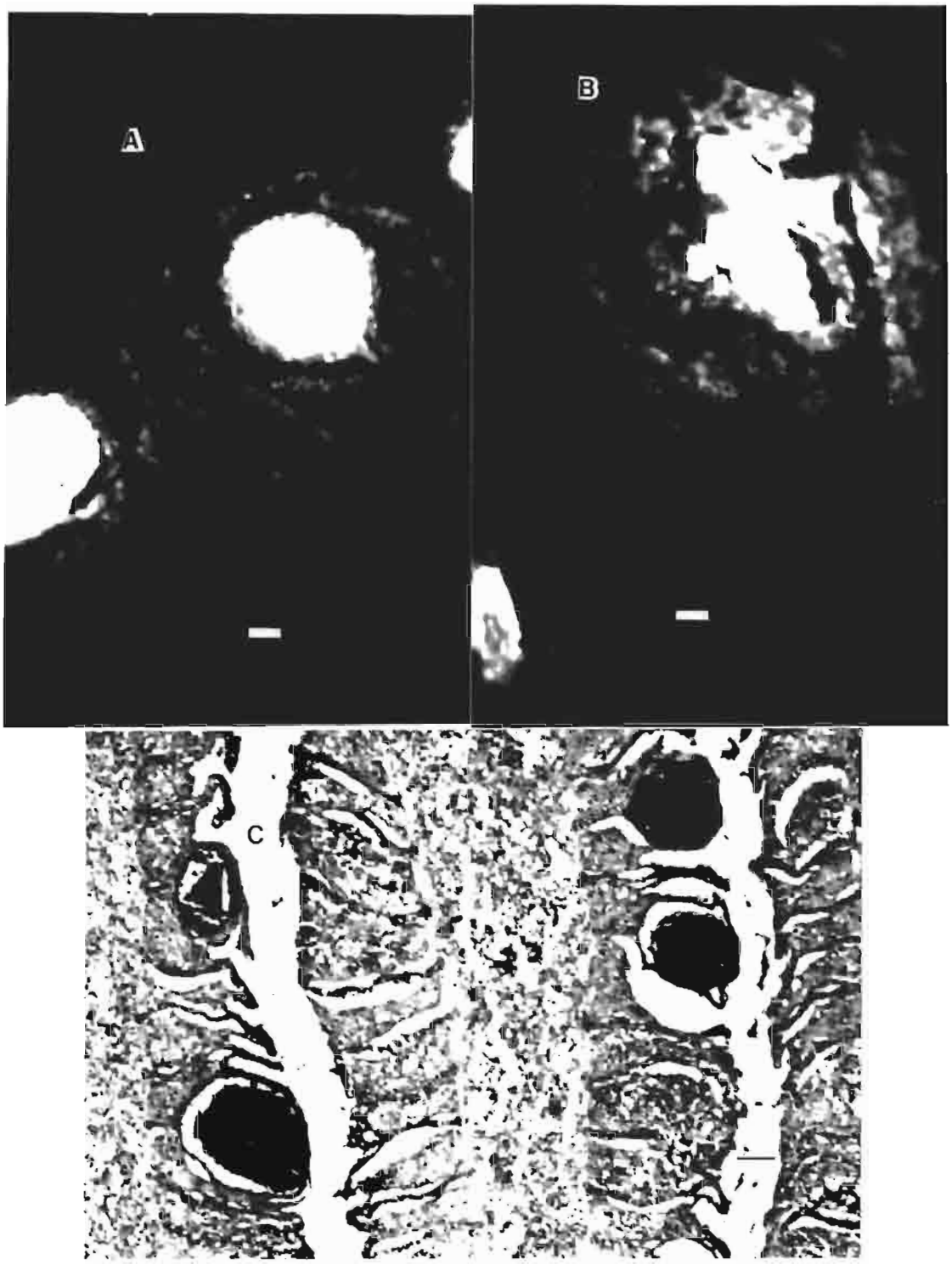

Figure 3.4. Immunostaining of *Henneguya exilis* spores in cysts from infected catfish gills using monoclonal antibodies to *H. exilis*. Indirect fluorescent antibody reactions on (A) formalin-fixed and (B) frozen tissues. (C) Immunoperoxidase reaction on formalin-fixed tissues. Bars=10μm

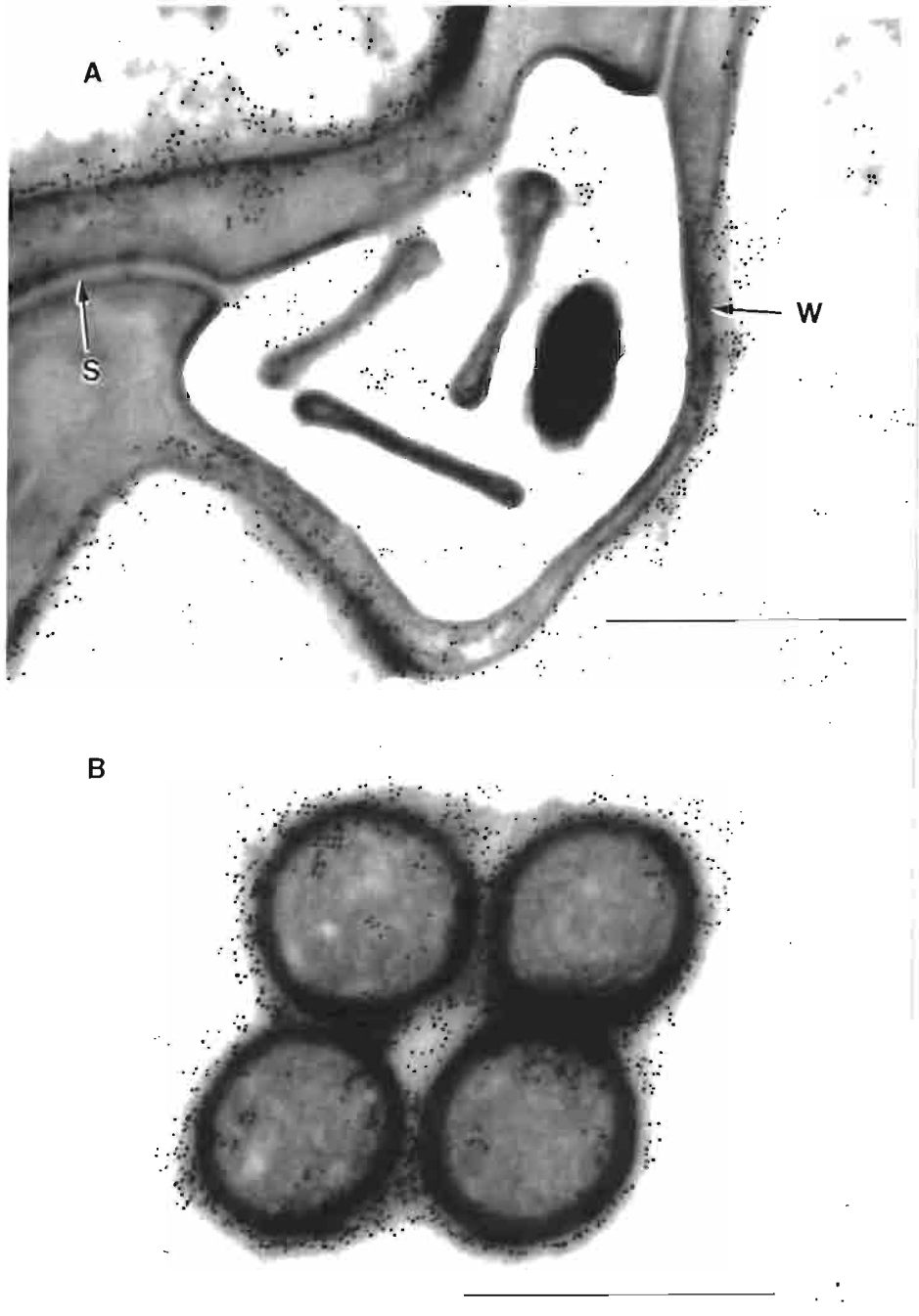

Figure 3.5. Electron micrographs of immunolabeling of *Henneguya exilis* spores using monoclonal antibodies to *H.exilis*. Gold particles (5 nm) on (A) walls of valves at the anterior pole and (B) the surface of cross-sections of tails. S=suture of valves; W=wall of valves; Bars= 0.5µm

Discussion

Immunohistochemistry by IFAT and IMPX tests on channel catfish tissues containing *Henneguya* sp. cysts showed that formalin-fixed as well as frozen tissues could be used. Formalin fixation did not appear to alter the antigenicity of specific epitopes on *Henneguya* sp. spores within the tissue cysts. The fact that formalin-fixed tissues were easier to process and analyze suggests that their use would be best for future immunohistochemistry studies of *Henneguya* sp. infected tissues.

Analysis of SDS-PAGE results showed differences in protein band patterns between *A. ictaluri* and *H. exilis*. Further studies are needed to better characterize the nature of the specific proteins noticed in this study with regard to the life stages of each organism. This would help in differentiating the two parasites.

All Mab tested in Western blots were positive for a 117 kD band from *H. exilis* spore antigens. However the reactivity of the polyclonal serum to more bands suggested that more antigenic products exist in the spores. The difference in number of bands of *Henneguya* spores recognized by the adsorbed and unadsorbed polyclonal sera suggested the existence of host antigen mimicry by the *Henneguya* spores. Lack of *A. ictaluri* spores in sufficient amounts precluded their use for Western blot experiments.

Monoclonal antibodies MH16c1, c3, and c10 could be used in diagnostic tests. Localization of antigenic determinants at the surface of the spores as shown by electron microscopy would make the detection of the parasite easier. Furthermore the Mab did not recognize any antigenic component of other myxosporeans and actinosporeans, and were able to detect single *Henneguya* sp. spores in histological sections. Thus these preliminary results indicated that specific Mab can be made to *H. exilis* that do not cross-react with other myxozoans. Some myxosporeans like *Ceratomyxa shasta* have even been shown to have stage-specific antigens (Bartholomew et al., 1989a). These Mab could also be used in studying the life cycle of the parasite and could help in the construction of an antigenic map of the parasite's surface (Adams et al., 1992).

The adsorbed and unadsorbed polyclonal sera reacted strongly to the unidentified myxosporean from bluegill fish, *A. ictaluri*, and *H. exilis* spores and *Henneguya* cysts in infected catfish tissues but failed to recognize the PGD organisms in infected tissue sections. This would suggest that antigens in *A. ictaluri* vary, depending on the life cycle stages of the organism.

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CHAPTER IV

SPECIFICITY AND CROSS-REACTIVITY OF IMMUNE SERA AND HYBRIDOMA ANTIBODIES TO VARIOUS SPECIES OF MYXOZOANS

Abstract

Monoclonal and polyclonal antibodies against *Henneguya exilis* and polyclonal antibodies against *Aurantiactinomyxon ictaluri* were produced. These antibodies were used to determine the relationship of these organisms to the myxosporeans: *Ceratomyxa shasta*, channel catfish proliferative gill disease (PGD) organism, and an unidentified myxosporean collected from skin nodules of bluegill fish, and the actinosporeans: *A. mississippiensis*, *Triactinomyxon brevis*, *Echinactinomyxon waylandi*, *Helioactinomyxon minutus*. Common antigenic determinants were found in indirect fluorescent antibody tests among all organisms analyzed. Genus-specific antigenic determinants were demonstrated by IFAT for *H. exilis* using monoclonal antibodies while the rabbit antiserum against *H. exilis* showed stage-specific antigenic determinants between the actinosporean and the myxosporean stages of the PGD organisms in channel catfish. Similar patterns of fluorescence were found between the myxosporeans: *H. exilis*,

Ceratomyxa shasta, the unidentified myxosporean, and the actinosporeans: *A. mississippiensis*, *T. brevis*, *E. waylandi*, *H. minutus* with minor differences in fluorescence on the polar capsules.

Introduction

A two-host life cycle similar to that described by Wolf and Markiw (1984) for whirling disease in salmonid fish has been demonstrated for proliferative gill disease (PGD) in channel catfish, *Ictalurus punctatus* (Burtle et al., 1991; Styer et al., 1991). It is now known that the life cycle of PGD involves an actinosporean *Aurantiactinomyxon ictaluri* that is liberated from an aquatic oligochaete host (*Dero digitata*), which after infecting the catfish is converted into a myxosporean cyst stage.

Several other actinosporean species from two families (Triactinomyxidae and Polyactinomyxidae) have also been isolated from *D. digitata* collected from ponds experiencing PGD outbreaks in their channel catfish populations (Bellerud, 1993; Pote and Waterstrat, 1993). The species isolated belonged to the genera *Aurantiactinomyxon*, *Triactinomyxon*, *Echinactinomyxon*, and *Helioactinomyxon*. Definitive classification of these actinosporeans, their relationship to PGD, and the complete life cycles of all these actinosporeans are currently unknown.

The purpose of this study was to determine the antigenic relationship among the myxosporeans: *Henneguya*

exilis, *Ceratomyxa shasta*, the channel catfish proliferative gill disease (PGD) organism, an unidentified myxosporean collected from skin nodules of bluegill fish, and the actinosporeans: *A. mississippiensis*, *T. brevis*, *E. waylandi*, *H. minutus*, and to evaluate the utilization of monoclonal and polyclonal antibodies in the diagnosis and examination of life cycles of myxozoan parasites.

Materials and Methods

Source of myxozoans and preparation of antigens

Henneguya exilis spores used for production of monoclonal and polyclonal antibodies (Mab and Pab) and for indirect fluorescent antibody tests (IFAT) were obtained and used in this study as previously described in Chapter III. Proliferative gill disease organisms used in IFAT were kept in tissue sections from organs collected in naturally or experimentally PGD infected catfish. Unidentified myxosporean spores were collected from bluegill fish (*Lepomis macrochirus*) skin nodules. Incidentally, these infected fish were obtained from channel catfish ponds in the Mississippi Delta. *Ceratomyxa shasta* spores from rainbow trout *Oncorhynchus mykiss* were provided by J.L. Bartholomew from Oregon State University, Corvallis, Oregon.

Actinosporean spores were prepared according to a previously described procedure (Pote et al., 1994). Briefly, mud was collected from ponds with confirmed PGD

outbreak in the catfish population. The mud was rinsed through a screen (300 μm aperature) to remove debris. Using a dissecting microscope, *D. digitata* were removed with a fine tip metal probe and placed in sterile spring water. The worms were rinsed in sterile spring water 3 times, individually transferred to 96-well plates with one worm per well, and covered with spring water. The plates were incubated at 22°C until actinosporeans spores were released by *D. digitata* into the well water. Spores were identified microscopically (Janiszewska, 1957; Marques, 1984; Bellerud, 1993) and pooled by species into conical 15 ml polystyrene test tubes. Five species from two families (Triactinomyxidae and Polyactinomyxidae) were collected and used for this study. They were: *A. ictaluri*, *A. mississippiensis*, *T. brevis*, *E. waylandi*, and *H. minutus*. Clean spores of *A. ictaluri* were enumerated, centrifuged at 450 x g and 10°C for 10 min and pellets of spores were collected and suspended to a concentration of 1×10^5 spores/ml for antigen preparation.

Antigenic solutions of concentrated *H. exilis* and *A. ictaluri* spores were prepared by sonication using a cell disruptor (Branson Sonifier cell Disruptor, Danbury, CT) at a power of 61 watts four pulses for 20 sec each. Spores were maintained on ice during sonication. Antigenic solutions of broken spores were divided into aliquots of 0.5 ml and kept at -20°C until use.

Production of monoclonal and polyclonal antibodies

Monoclonal antibodies (MAb) were produced against *H.exilis* (Chapter III) and polyclonal antibodies (Pab) were produced against both *H. exilis* (Chapter III) and *A. ictaluri*. Procedures for productions of Mab and Pab, and for the adsorption of the Pab in live catfish were identical to those described in Chapter III. Immunization dose of *A. ictaluri* was 2 x 10^4 sonicated spores.

Indirect fluorescent antibody test

Monoclonal and polyclonal (1/20) antibodies with the exception of the polyclonal antibodies against *A. ictaluri*, used in this experiment were those described in Chapter III. The IFAT technique was identical to the procedure outlined in Chapter III. Immunofluorescence tests included controls of negative hybridoma fluid, sera from the mice immunized for the production of hybridomas, and sera from nonimmunized rabbits.

Results

Three hybridomas specific for *H. exilis* spores were used in the cross-reactivity study. All three Mab reacted strongly to *H. exilis* spores or *H. exilis* cysts in catfish tissue sections when tested with IFAT (Table 4.1 and Figure 4.1). They also reacted to immature stages of *H. exilis* in gill sections. Individual *H. exilis* spores showed fluorescence on valves but there was little fluorescence on

the polar capsules or in the sporoplasm. However, after the polar filaments were extruded they were fluorescent. The three Mab to *H. exilis* did not react to any of the other myxosporeans or actinosporeans used in this study.

Adsorption of rabbit antisera in live catfish reduced background staining in IFAT on tissue sections to negligible levels. All polyclonal antibodies against *H. exilis* and *A. ictaluri* reacted more positively to spores and tissue cyst stages than did Mab produced to *H. exilis* spores when a reaction occurred (Figure 4.2). The Pab showed patterns of fluorescence on the *H. exilis* spores similar to the patterns observed for the Mab. The adsorbed antiserum against *H. exilis* reacted positively in IFAT to *A. ictaluri*, *A. mississippiensis*, *T. brevis*, *E. waylandi*, *H. minutus* spores, *C. shasta* and the unidentified myxozoan spores (Figure 4.2 and 4.3) but reacted negatively to the PGD organism in fish tissue. The adsorbed antiserum against *A. ictaluri* reacted positively using IFAT to all actinosporeans and myxosporeans tested (Table 4.1). In the actinosporeans, fluorescence was localized in the epispore including the three appendages and the polar capsules. The sporoplasms of *A. ictaluri*, *A. mississippiensis* (Figure 4.2B and C), and *E. waylandi*

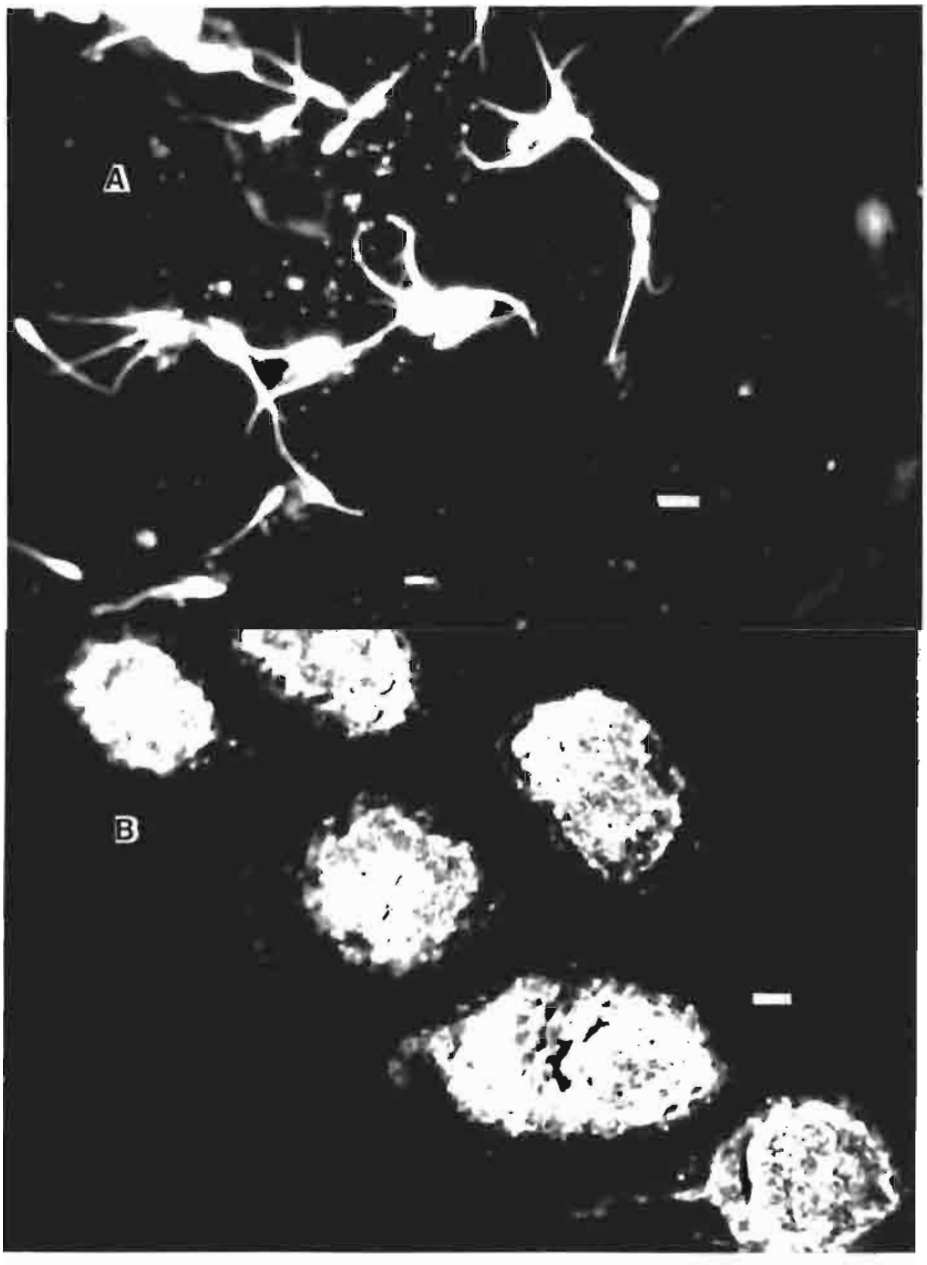


Figure 4.1. Indirect fluorescent antibody reaction of *Henneguya exilis* spores in (A) pure preparation or in (B) cysts from catfish gill tissue section probed with monoclonal antibody to *H. exilis*. Bars=10μm

(Figure 4.3 B) were less fluorescent. The unidentified myxosporean and *H. minutus* showed nonspecific fluorescence (Figure 4.3 E and C). *Ceratomyxa shasta* had fluorescence primarily in the walls of the valves (Figure 4.3 D). In PGD infected fish tissues, fluorescence was located in the envelopes of cysts and trophozoites inside the PGD cysts.

Negative hybridoma supernatant and nonimmunized rabbit sera did not react with any of the myxozoans (Figure 4.4).

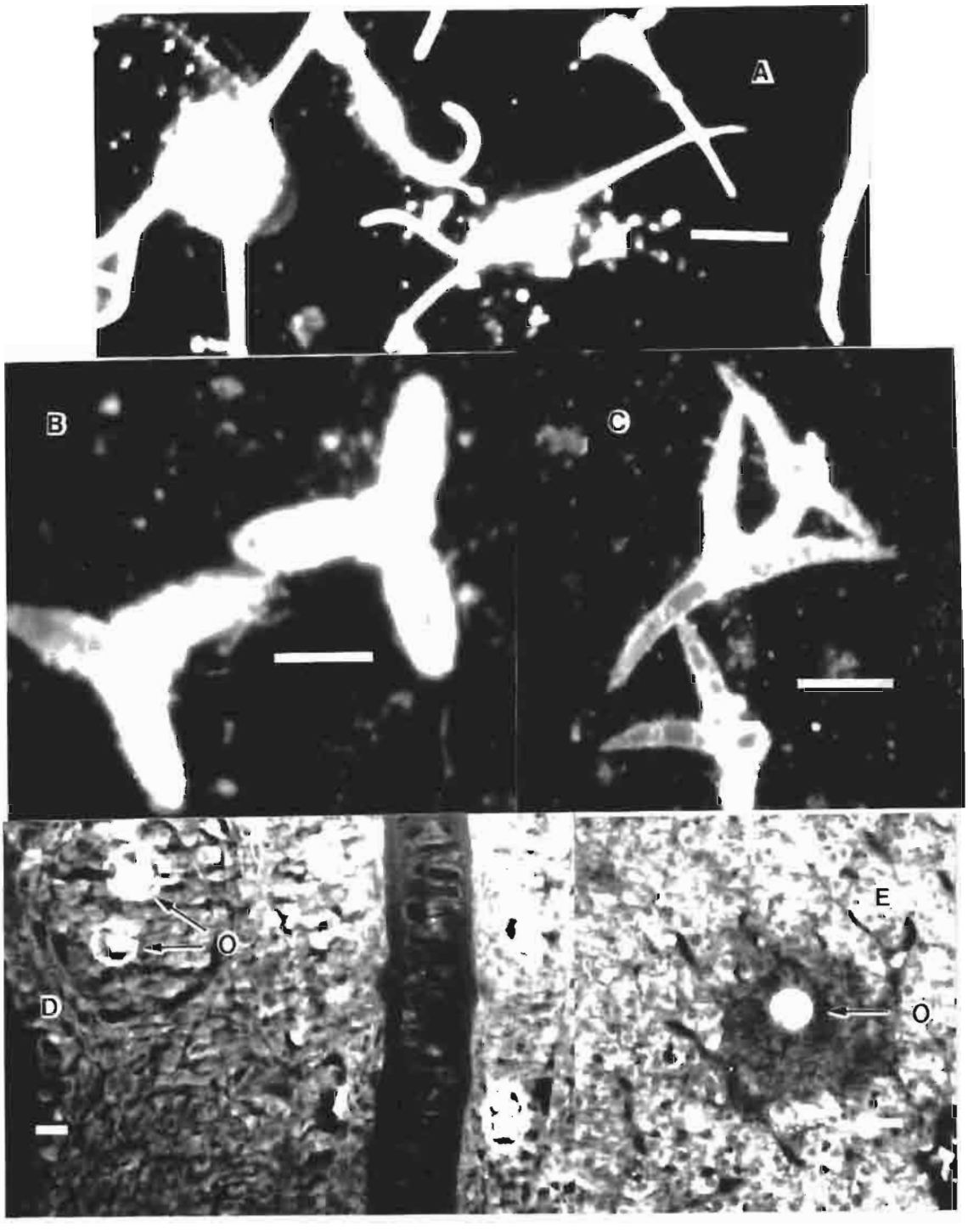


Figure 4.2. Indirect fluorescent antibody reactions of pure spore preparations of (A) *Henneguya exilis*, (B) *Aurantiactinomyxon ictaluri*, (C) *A. mississippiensis* probed with adsorbed antisera to *H. exilis* or *A. ictaluri*, and of (D) PGD organisms in catfish gills and (E) liver tissue sections probed with adsorbed antiserum against *A. ictaluri*. O=organism. Bars=10μm

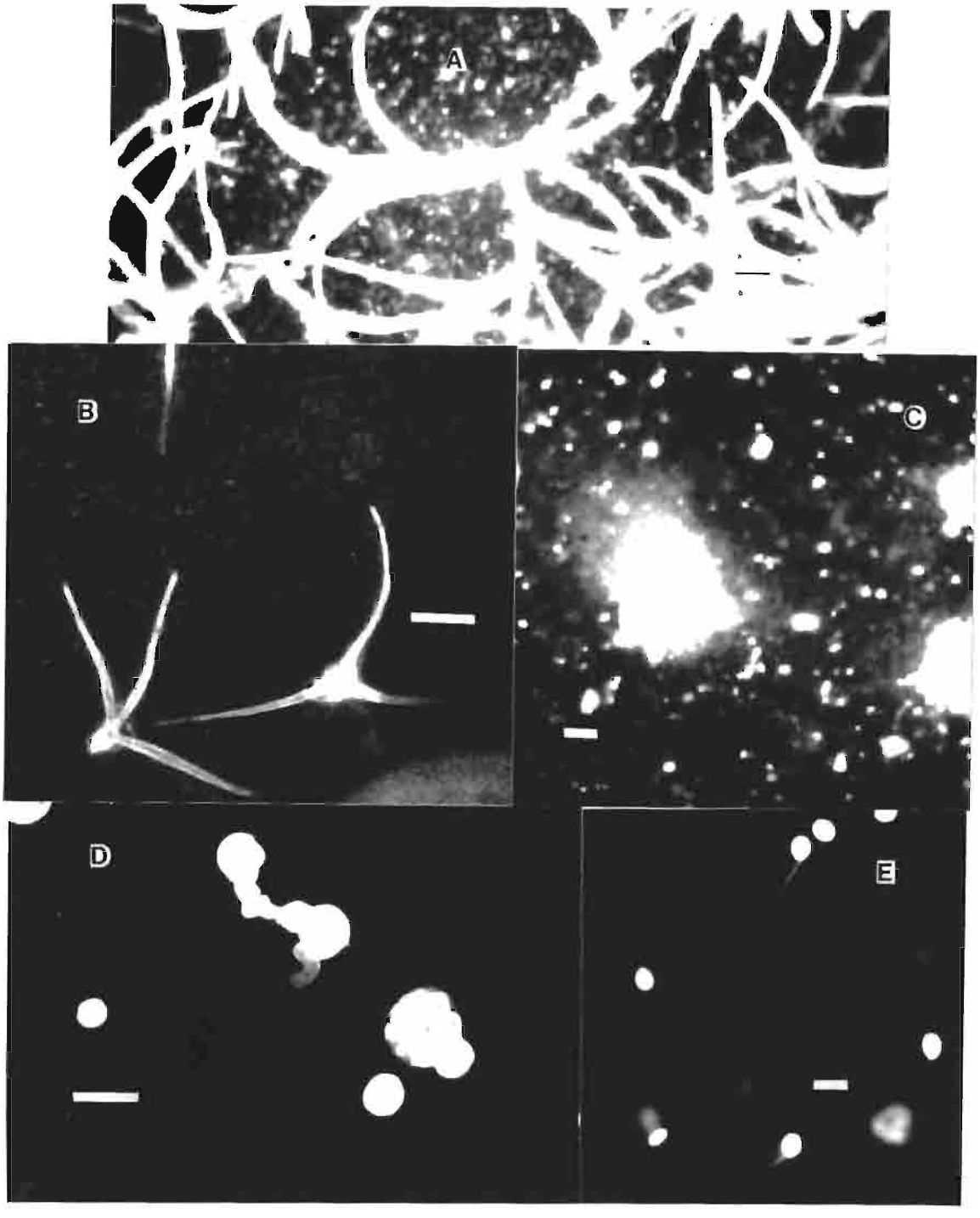
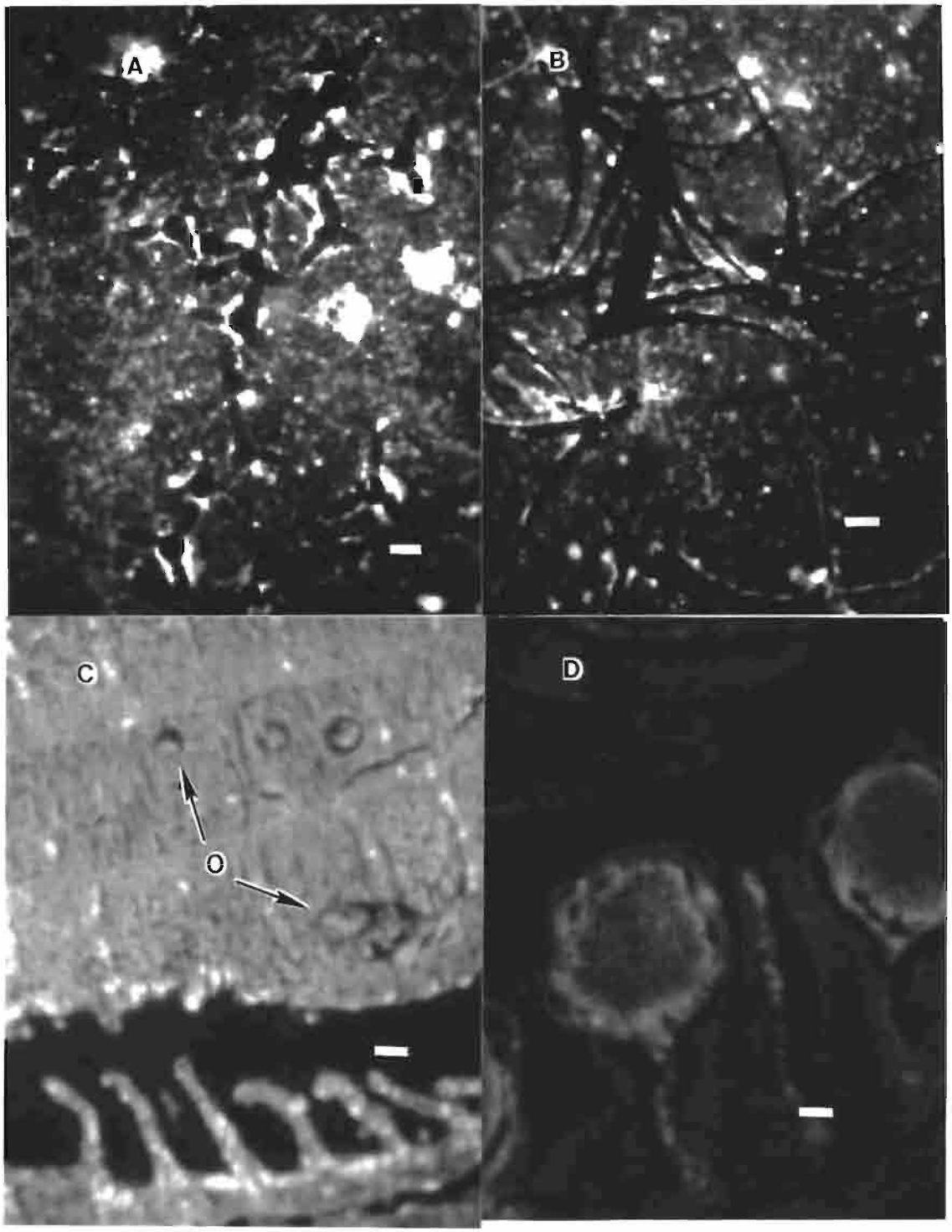


Figure 4.3. Indirect fluorescent antibody reactions using adsorbed antisera to *Henneguya exilis* or *Aurantiactinomyxon ictaluri* on pure spore preparations of (A) *Triactinomyxon brevis*, (B) *Echinactinomyxon waylandi*, (C) *Helioactinomyxon minutus*, (D) *Ceratomyxa shasta*, (E) and an unidentified myxosporean collected from skin nodules of a bluegill fish. Bars=10μm



4.4. Negative indirect fluorescent antibody reactions using adsorbed nonimmunized rabbit sera on pure spore preparations of (A) *Aurantiactinomyxon ictaluri*, (B) *Triactinomyxon brevis*, and on (C) PGD organisms and (D) *Henneguya exilis* spores in cysts from catfish gills. O=organism. Bars=10μm

Table 4.1. Cross-reactivity of antibodies with different myxosporeans and actinosporeans.

		Monoclonal Abs to *Henneguya exilis*	Polyclonal Abs to *Henneguya exilis*	Polyclonal to *Aurantiactinomyxon ictaluri*
Myxosporeans	*H. exilis* (pure spores)	+	+	+
	H.exilis(in tissue sections)	+	+	+
	Unknown myxosporean	-	+	+
	PGD organism in tissues	-	-	+
	Ceratomyxa shasta	-	+	+
Actinosporeans	*Aurantiactinomyxon ictaluri*	-	+	+
	A. mississipiensis	-	+	+
	Triactinomyxon brevis	-	+	+
	Echinactinomyxon waylandi	-	+	+
	Helioactinomyxon minutus	-	+	+

Discussion

Myxosporean and actinosporean antigens have been detected by serological techniques such as IFAT and Ouchterlony immunodiffusion tests (Halliday, 1974; Pauley, 1974; Markiw and Wolf, 1978; Amandi et al., 1985; Markiw, 1989). Markiw (1989) demonstrated cross-reactions between the myxosporean and actinosporean stages in the whirling disease life cycle using direct fluorescent antibody test. In this study, the strong serological responses of rabbit antisera against both the myxosporean *H. exilis* and the actinosporean *A. ictaluri* to almost all myxozoans tested (*H. exilis*, *C. shasta*, the channel catfish proliferative gill disease (PGD) organism, the unidentified myxosporean collected from skin nodules of bluegill fish, *A.ictaluri*, *A. mississippiensis*, *T. brevis*, *E. waylandi*, *H. minutus*) indicated that antigenic determinants are shared. *Aurantiactinomyxon ictaluri* was shown to be serologically related to its corresponding myxosporean in catfish organs infected with PGD. However the antiserum against *H. exilis* reacted positively to *A. ictaluri* spores but negatively to the PGD organisms in catfish tissue sections suggesting the presence of different antigenic determinants in the myxosporean stages found in PGD. Genus-specific antigenic determinants were revealed by Mab to *H. exilis* which were specifically positive to only *H. exilis* in mature or immature forms.

Monoclonal antibodies against *H. exilis* offers a possible tool for the diagnosis and epizootiological study of this parasite in channel catfish populations. Rabbit antiserum against *A. ictaluri* can be used in the study of the early developmental stages of PGD.

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CHAPTER V

PORTALS OF ENTRY AND SYSTEMIC LOCALIZATION OF PROLIFERATIVE GILL DISEASE ORGANISMS IN CHANNEL CATFISH (*ICTALURUS PUNCTATUS*, RAFINESQUE)

Abstract

Immunized rabbit serum adsorbed in live catfish was used in indirect fluorescent antibody test (IFAT) to detect the myxozoan parasite developmental stages responsible for proliferative gill disease in channel catfish (*Ictalurus punctatus*). Specific pathogen free fingerlings were experimentally infected with *Aurantiactinomyxon ictaluri* and necropsied 24, 48, 72, and 96 hours post infection. At 24 h post-exposure intracellular forms of sporozoites were observed primarily in gastric epithelium and submucosa but also in skin and buccal cavity. Ovoid unicellular organisms were detected in heart and blood vessels of the liver. Small fluorescent intracellular inclusions were often seen in the parenchyma of spleen, head and trunk kidneys. From 48 to 72 h after exposure, fewer fluorescent organisms were located in all organs than was observed at 24 h. These organism appeared to be degenerating except for those in the gills which were multinucleated. By 96 hours post-

infection, the organisms could not be detected in fish tissues with the exception of multicellular trophozoites remaining in the gills which appeared to be a privileged site of development. Throughout the entire 96 hour period of study, no form of the organism was detected in the brain. Infected tissue sections treated with non-immune rabbit serum and non-infected tissue sections treated with immune rabbit sera all showed negative results by IFAT.

Introduction

Proliferative gill disease (PGD), also called hamburger gill disease, is a serious parasitic problem in the commercial channel catfish (*Ictalurus punctatus*). The disease is characterized by branchial inflammation, epithelial hyperplasia, lysis of filamental cartilages, lamellar fusion, and the presence of myxozoan trophozoites usually in the gills (MacMillan et al., 1989; Burtle et al., 1991).

Several myxosporeans including *Henneguya* sp., *Sphaerospora* sp., and *Aurantiactinomyxon* sp. had been previously implicated as etiologic agents in the life cycle of the PGD organism (Bowser et al., 1985; MacMillan et al., 1989; Hedrick et al., 1990; Burtle et al., 1991; Styer et al., 1991). However recent work has demonstrated that the life cycle of the PGD organism is similar to that described by Markiw and Wolf (1983) and Wolf and Markiw (1984). This life cycle demonstrated that the causative myxozoan in

salmonid whirling disease had a 2-host life cycle involving fish infected with a myxosporean and an aquatic oligochaete infected with an actinosporean. This lead investigators to examine the oligochaete populations in ponds with PGD-positive fish for the presence of actinosporeans.

In early research, PGD had been produced experimentally by exposure of catfish to mud from ponds containing fish with PGD (Bowser et al., 1985; MacMillan et al., 1989). Burtle et al. (1991) demonstrated the consistent presence of an oligochaete *Dero digitata* in ponds during outbreaks of PGD. Finally, pure *A. ictaluri* isolated from *D. digitata* (Pote et al., 1992) or infected *D. digitata* worms shedding this actinosporean (Styer et al., 1991) were successfully used to experimentally reproduce PGD in channel catfish.

Pote and Waterstrat (1993) showed that *Aurantiactinomyxon* sp. produced a motile amoeba-like stage upon its exposure to catfish gill filaments. However the role of polar capsules and filaments, the complete life cycle of this actinosporean, and the definitive taxonomy of all actinosporeans isolated from *D. digitata* are currently unknown. In addition to the gills, PGD trophozoites have been demonstrated in liver, head and trunk kidneys, spleen, and brain of infected catfish (Bellerud, 1993; Thune, 1993). It is not known however, the point of entry of the parasite, the morphology of the early stages in the fish or how the organism is transported systemically. The purpose of this

research was to demonstrate portals of entry of *A. ictaluri* and detect the early stages of this parasite and its dissemination in the channel catfish.

Materials and Methods

Preparation of spores and infection of catfish

Aurantiactinomyxon ictaluri spores were prepared according to a modified procedure described by Pote et al. (1994). Briefly, mud was collected from a pond with a confirmed PGD outbreak in the catfish population and rinsed through a screen (300 μm aperature) to remove debris. A dissecting microscope was used to examine material captured on the screen and isolate *D. digitata*. The worms were placed in sterile spring water, rinsed with three exchanges of sterile spring water and then they were individually transferred to wells of 96-well plates, and covered with spring water. The plates were incubated at room temperature until actinosporean spores were released by the infected worms. Spores identified as *A. ictaluri* (Pote and Waterstat, 1993; Marquès, 1984) were collected together into conical 15 ml polystyrene test tubes and counted.

Specific pathogen free (SPF) channel catfish fingerlings were placed in three 19 l tanks. Twenty fingerlings each were placed in Tanks 1 and 2, and ten in tank 3. Prior to infection the water was lowered in all tanks (to approximately 7.5 cm). *Aurantiactinomyxon ictaluri*

spores were added to Tank 1 and 2 (2,000 spores/tank); Tank 3 served as a non-infected control. Six hours later, the water level was raised in all tanks to about 27 cm. At 24, 48, 72, and 96 hours post infection, five fish each were collected from Tank 1 and 2, and one fish from Tank 3. All fish were necropsied and tissues placed in 10 % neutral formalin for at least 48 hours before routine histopathology processing. The gills, stomach, intestines, head and trunk kidneys, liver, spleen, heart, skin, muscle, and brain were collected from each fish and placed in 10% buffered formalin. Paraffin-embedded histological sections of all tissues (4 μm thick) were either stained with Mayer's haematoxylin and eosin (H and E) and examined by light microscopy (100 or 400 x magnification) or deparaffinized, rehydrated, rinsed in distilled water, and used in immunocytochemistry.

Production of polyclonal antiserum and its use in IFAT

Clean spores of *A. ictaluri* were collected from *D. digitata* (Pote et al., 1994) and adjusted to a concentration of 1 x 10^5 spores /ml. Polyclonal antibodies to *A. ictaluri* were made as described in previous chapter. Briefly, concentrated *A. ictaluri* spores were sonicated and used as antigen to immunize rabbit. The antigen was diluted with sterile distilled water to approximate the delivery of 2 x 10^4 spores of *A. ictaluri* /dose. Immune and non-immune rabbit sera were adsorbed in live catfish according to a

modified procedure described by Jensen and Hedrick (1987). Adsorbed and non-adsorbed sera, and non-injected SPF catfish serum were all tested for specificity by IFAT on pure *A. ictaluri* spores and on histopathology confirmed PGD infected gill sections.

Polyclonal antibodies were incubated 30 min at 37°C with air-dried *A. ictaluri* spores or tissue sections on glass microscope slides. All specimens were fixed in cold acetone for 15 min. Paraffin embedded tissue sections were deparaffinized, rehydrated, and rinsed in distilled water prior to use (Haines and Chelack, 1991). Specific antibodies were detected using goat fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulins (Sigma, St. Louis, MO). Washes were done with phosphate buffered saline (0.01 M phosphate and 0.4 M sodium chloride PBS) pH 8.0. Coverslips were mounted using Vectashield mounting media (Vector Laboratories, Burlingame, CA). Spores and tissue sections were examined using a Zeiss standard microscope (Carl Zeiss, Oberkochen, West Germany) equipped with epi-fluorescence condenser and a 100 W xenon arc lamp.

Results

At 24 hours post-exposure to the actinosporean *A. ictaluri* small intracellular forms of sporozoites were observed in gastric epithelial cells and gastric submucosa (Figure 5.1 A and B). Similar forms of fluorescent

organisms were also seen in the epithelia of the skin and buccal cavity, particularly at the base of the gills (Figure 5.1C and D). Ovoid unicellular stages were visible in the heart and blood vessels of the liver (Figure 5.2A). All the gills examined demonstrated the presence of these unicellular forms but some ovoid or round multicellular forms of trophozoites with 2 to 4 nuclei were observed in the stroma of some of the gill filaments (Figure 5.2B and C). Small fluorescent intracellular inclusions were often seen in the parenchyma of spleen and head and trunk kidneys. Amorphous aggregates of very bright fluorescent material were observed in the lumen of the intestines or coating the intestinal epithelia (Figure 5.2D).

From 48 hours to 72 hours after exposure, unicellular forms appeared in deeper strata of the stomach composed of gastric glands (Figure 5.3C). Fewer intracellular forms were located in the skin and the mouth than was observed at 24 h. Heart and liver blood vessels also showed less unicellular ovoid forms than seen at 24 h. The number of organisms appeared to decrease in the gills with time but those organisms remaining were either multinucleated or degenerating (Figure 5.3A and B). These multinucleated forms of trophozoites were located in gill filament stroma along the cartilage. At 72 hours, most gills showed very bright fluorescent forms without obvious nuclei. Inclusions in hematopoietic cells of the kidneys and spleen parenchyma

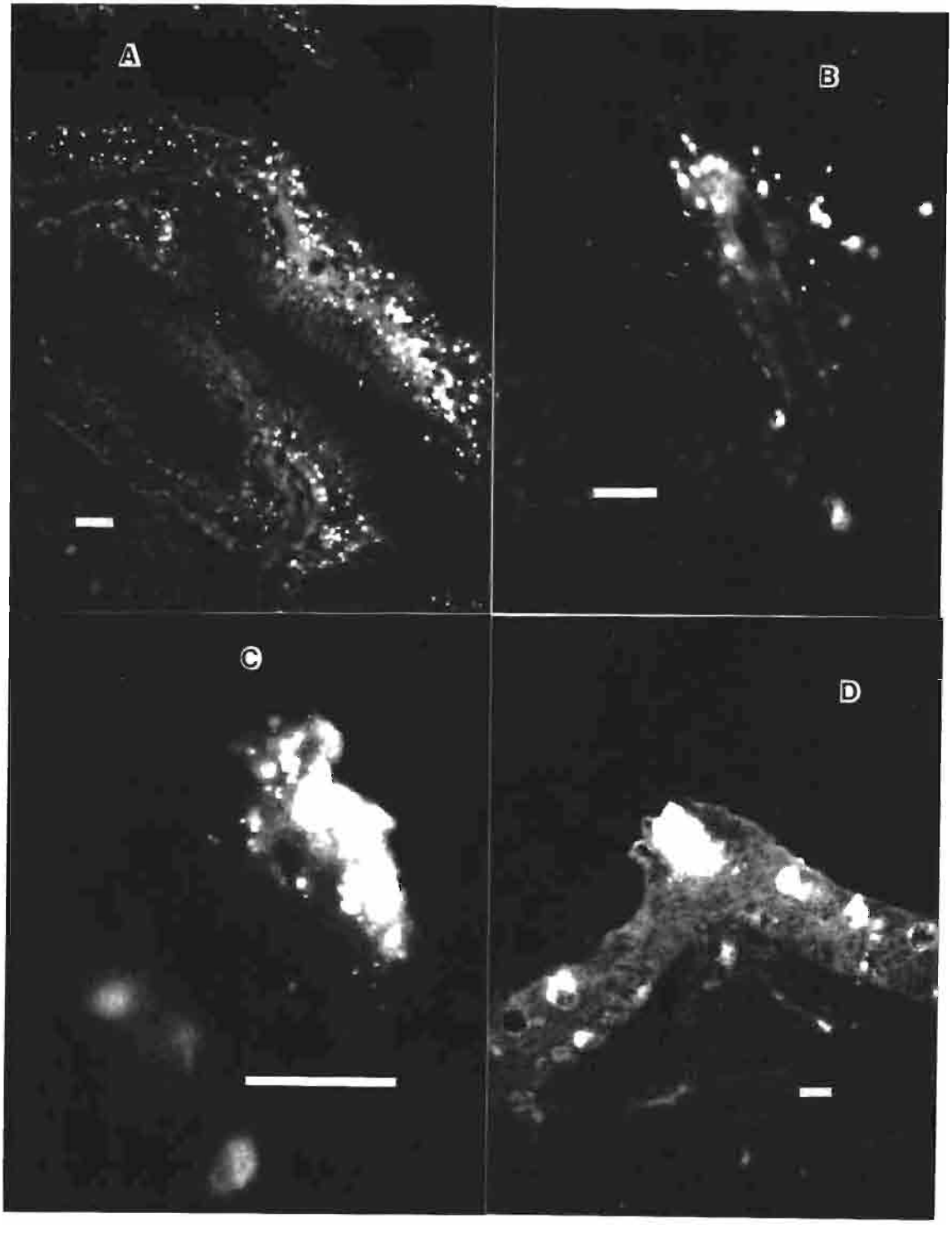

Figure 5.1. Fluorescent proliferative gill disease organisms in (A) stomach epithelium and (B) submucosa, in (C) skin, and in (D) base of gills of SPF catfish, 24 h after artificial infection with *Aurantiactinomyxon ictaluri*. Bars=10μm

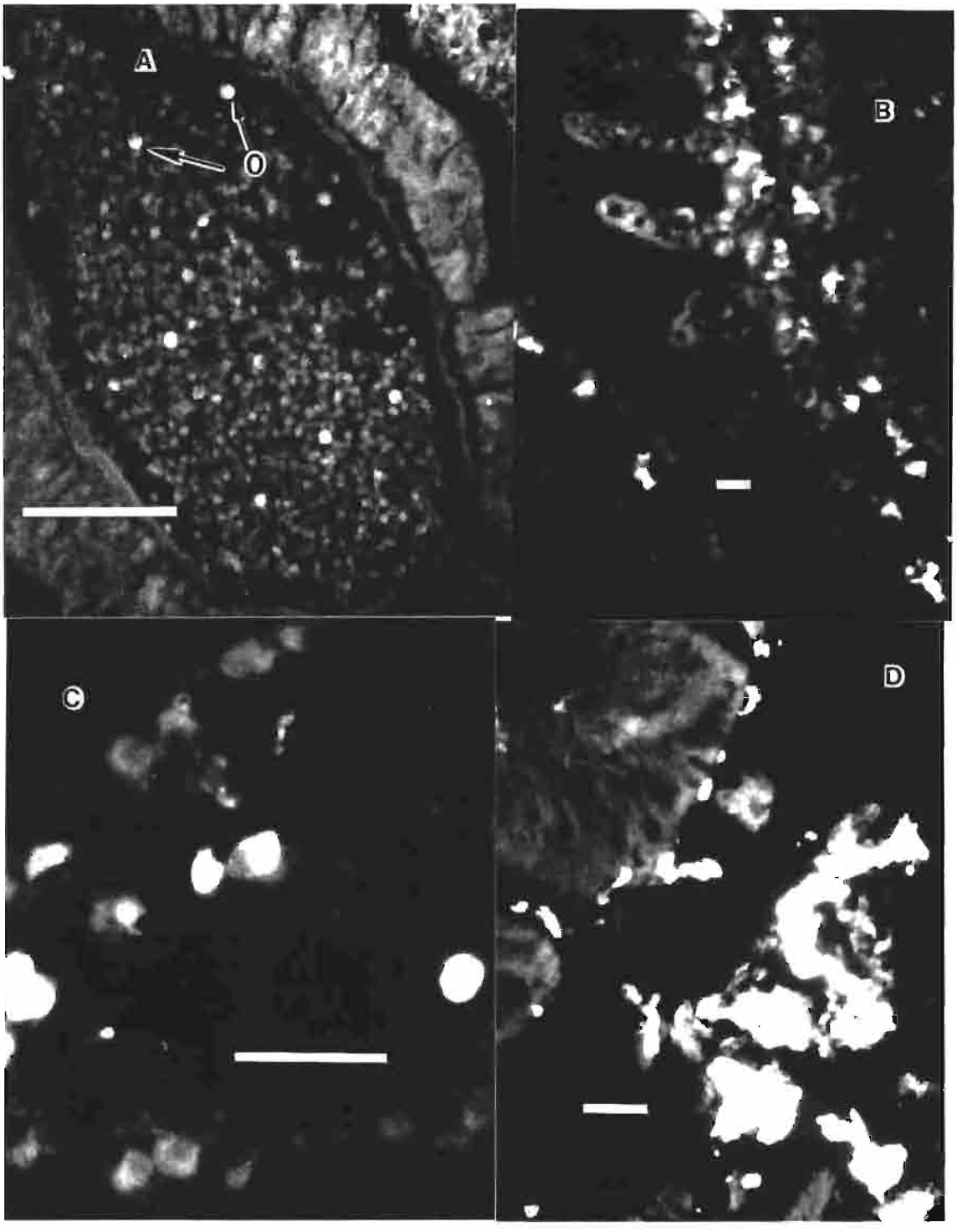

Figure 5.2. Fluorescent proliferative gill disease organisms in (A) the liver blood vessel, (B and C) gill stroma, and in (D) lumen of the intestines (epispores) of SPF catfish, 24 h after artificial infection with *Aurantiactinomyxon ictaluri*. O=organism. Bars=10μm

were less numerous and the fluorescent intensity had decreased when compared to organisms seen at 24 hours.

At 96 hours post-exposure, most of the fluorescent organisms had disappeared from the stomach, heart, liver, spleen, and kidneys. Only multinucleated trophozoites were observable in the stroma of gill filaments (Figure 5.4). Furthermore these organisms were less numerous but more focally developed. All brains examined were negative during the entire study period. Negative results were obtained by IFAT for all specimens examined with non-immunized rabbit serum and for all non-infected tissue sections.

Histopathology of tissue sections of all organs and tissues stained with H and E showed negative results except for the gills. Few cases of inflammatory and proliferative lesions in the gills were detected at 24 hours post exposure. The first time an organism was seen was at 48 hours after exposure in a single case and it was an uninucleate form located in the stroma of gill filament. Multinucleated trophozoites were found in the gills at 72 hours and 96 hours after infection.

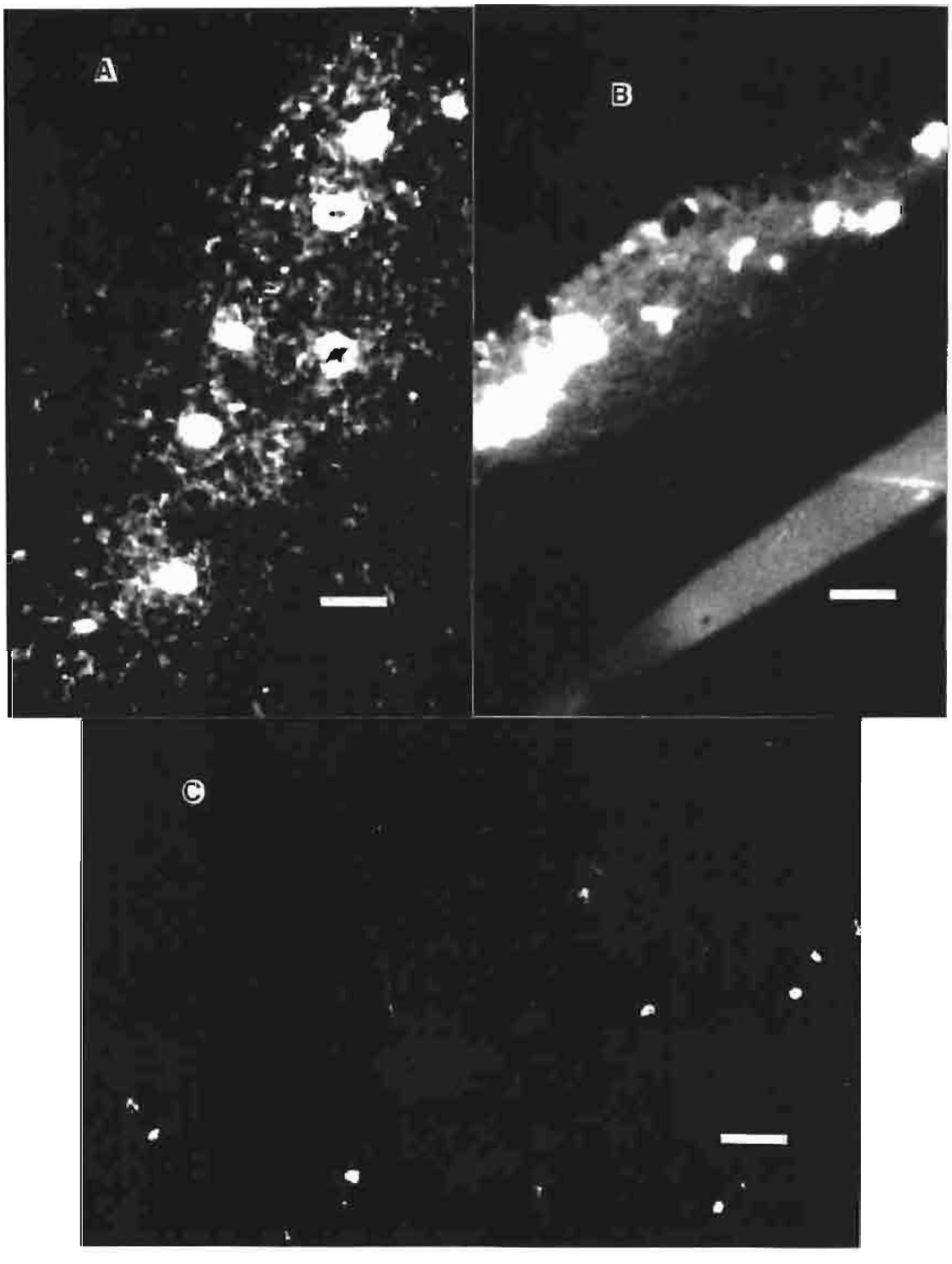

Figure 5.3. Fluorescent proliferative gill disease organisms in (A and B) gills and in (C) gastric glands of SPF catfish from 48 to 72 h after artificial infection with *Aurantiactinomyxon ictaluri*. Bars=10µm

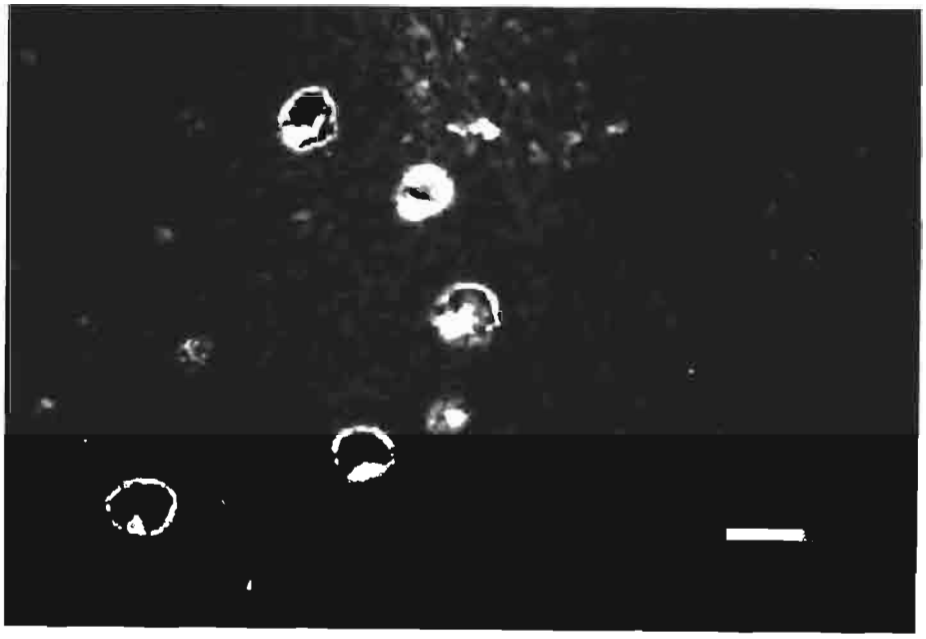

Figure 5.4. Fluorescent proliferative gill disease organisms in gills of SPF catfish, 96 h after artificial infection with *Aurantiactinomyxon ictaluri*. Bars=10µm

Discussion

This experiment demonstrated that IFAT could be used to detect early stages of PGD organisms in infected channel catfish tissues not normally observed by the more traditional histopathology methods employing H and E staining with bright field microscopy. Using IFAT, organisms were detected 24 hours after infection. Pure *A. ictaluri* spores and SPF catfish fingerlings were used in this experiment to prevent or reduce the chances of cross-reactivity of the rabbit polyclonal serum with anything other than the PGD organisms. Specificity of the polyclonal serum was further enhanced by adsorption in live catfish. Since organisms were already visible in several locations in the catfish at 24 h post infection, future studies should examine what happens to these stages prior to 24 hours post exposure. The present study also corroborated the findings that the actinosporean *A. ictaluri* shed by *D. digitata* causes PGD in catfish (Styer et al., 1991; Pote et al., 1992).

Recent studies by Pote and Waterstrat (1993) demonstrated the production of a motile stage of *A. ictaluri* upon exposure to channel catfish gills. Although the mechanism of penetration of the sporoplasm into the host and the role of polar capsules and filaments remains unknown, the present study suggests that multiple sites of entry are possible based on the observation of similar intracellular

fluorescent forms in the skin, the base of the gills in the buccal cavity, and the stomach. The stomach seemed to be the major site of penetration of the sporozoites as indicated at 24 h. Fluorescent material found in the lumen of the intestines may have been the epispores from actinosporeans in the intestinal tract. This could have indicated that motile stages (Pote and Waterstrat, 1993) were freed upon contact with fish tissues in the stomach and intestinal tract. After penetration into the host tissues, sporozoites of PGD appeared to move or were transported through deeper layers to blood vessels where they were then transported to the heart. This was evident by the presence of unicellular forms in the heart and liver blood vessels in association with red blood cells. The blood circulation could have been responsible of further dissemination of sporozoites to organs such as the spleen, kidneys, liver, and gills where organisms were found at 24 h post-exposure. The results of this study are similar to the work by Markiw (1989) which showed multiple portals of entry for a myxozoan, *Myxobolus cerebralis*, into salmonid fish.

Organisms were degenerating or decreasing in number at 96 h in the stomach, skin, liver, spleen, kidneys and heart. This indicated that these parasitic stages might have been destroyed by the host non-specific defense mechanism (Ellis, 1978). The destruction of organisms is most likely explained by the typical inflammatory response and lesions

associated with this disease demonstrated in traditional histopathology studies (MacMillan et al., 1989). The gills seemed to be privileged sites of development for the PGD myxosporeans since intact multicellular trophozoites were still observed at 96 hours post-exposure but were degenerated or non-existent in other organs.

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CHAPTER VI

PROLIFERATIVE GILL DISEASE (PGD) IN CHANNEL CATFISH *ICTALURUS PUNCTATUS* AFTER ADMINISTRATION OF DEXAMETHASONE OR PARENTERAL INJECTIONS OF *HENNEGUYA EXILIS* AND *AURANTIACTINOMYXON ICTALURI*

Abstract

Two experiments were conducted to determine the response of specific-pathogen-free (SPF) catfish (*Ictalurus punctatus*) to *Aurantiactinomyxon ictaluri* challenges after injection of sonicated *Henneguya exilis*, *A. ictaluri* or dexamethasone. Fish (n= 10/ treatment group) were infected with 15,000 *A. ictaluri* spores one week after injection of antigenic solutions of *H. exilis* (1.0 x 10^5 spores/fish) and *A. ictaluri* (2.0 x 10^3 spores/fish), and 1 to 2 weeks after dexamethasone injection. Fish injected with sonicated *H. exilis* and *A. ictaluri* were necropsied one week post infection and dexamethasone-injected fish were necropsied every week for 3 weeks post infection. In general, lesions and PGD organisms detected in gills using histopathology were similar in both experiments. However, in experiment 1 (injection of myxozoan antigen), 30% of the positive control

fish had organisms and lesions in non-gill organs versus 45% for the fish injected with *H. exilis* and 21% for the fish injected with *A. ictaluri*. These percentages were not statistically different among the 3 treatment groups, however the large values found for the 95% confidence intervals for the differences in the proportions suggested that biologically significant differences might have been detected with greater numbers of fish per treatment group. These results may have indicated immunosuppression to *A. ictaluri* with *H. exilis* immunization and protection with the *A. ictaluri* immunization. In experiment 2, those fish administered dexamethasone for 2 weeks prior to challenge had significantly higher ($p < 0.05$) incidence of non-gill lesions and organisms (95%) as compared to the fish administered dexamethasone for 1 week (47.4%) or the positive control fish (60%). Furthermore the lesions and organisms in the fish organs were intact longer in this group. During both studies the fish remained asymptomatic and no mortalities were observed. Using indirect fluorescent antibody test (IFAT) to *A. ictaluri* no antibodies were detected in pooled sera collected from all groups of catfish.

Introduction

Proliferative gill disease (PGD) of channel catfish (*Ictalurus punctatus*) is associated with significant mortalities and is characterized by myxozoan stages in the

gill causing swelling and necrosis of the gills. The development of severe granulomatous branchitis is indicated by thickening of gill filaments due to a mixed cellular infiltrate and fusion of gill lamellae following epithelial hypertrophy and hyperplasia. Mixed cell populations in the gills are comprised of lymphocytes, neutrophils, and macrophages. Necrosis of the gill cartilage is usually evident and small basophilic parasites are randomly located within the thickened gill filaments (MacMillan et al., 1989; Burtle et al., 1991; Styer et al., 1991). Proliferative gill disease organisms have also been observed in liver, kidney, spleen, and brain (MacMillan et al., 1989; Styer et al., 1991; and Bellerud, 1993).

In early experiments PGD was produced in catfish by exposure to mud from ponds containing fish with PGD (Bowser et al., 1985; MacMillan et al., 1989). It is now believed that a 2-host life cycle involving fish and an aquatic tubificid oligochaete similar to that demonstrated for the myxozoan associated with whirling disease in salmonids (Wolf and Markiw, 1984) occurs in PGD in channel catfish. Recently studies have shown that catfish exposed to pure *Aurantiactinomyxon ictaluri* (Myxozoa: Actinosporea) isolated from *Dero digitata* (Pote et al., 1992) or *A. ictaluri* infected *D. digitata* worms (Styer et al., 1991) had the myxozoan organisms associated with PGD. The point of entry into catfish of these organisms is not known, however recent

research has shown that a motile stage of *A. ictaluri* is induced after exposure to channel catfish gill filament which might play a role in the entry of these organisms into the fish (Pote and Waterstrat, 1993).

It is theorized that humoral and cell-mediated responses during myxosporean infections are mild because the parasite antigenically mimics host tissues (Woo, 1992). Griffin and Davis (1978) detected antibodies against *Myxosoma cerebralis* in salmonid fish yet in another study no circulating antibodies were found after exposure to the same parasite (Halliday, 1974; Pauley, 1974) or to the myxozoan *Ceratomyxa shasta* in salmonids (Bartholomew et al., 1989). The presence of an intense cellular response with infiltration of macrophages and lymphocytes and failure of complete sporogony in infected hosts are unusual in myxosporean infections unless the parasite is in an abnormal site or aberrant host (Woo, 1992; MacMillan et al., 1989). However in the case of proliferative kidney disease (PKD) in rainbow trout (*Oncorhynchus mykiss*) the fact that immunosuppression with cortisone promoted the earliest stages to PKD organism sporogony suggested that the host's immune system may play a role in the incomplete sporogony (Kent and Hedrick, 1987). In higher vertebrates corticosteroids have been shown to enhance the development of parasites in normal as well as abnormal hosts (Dale et al., 1973; Bach, 1976). This paper investigated the effect

of injections of dexamethasone or immunization with *H. exilis* or *A. ictaluri* spores in catfish and the subsequent challenge with *A. ictaluri* on the pathogenesis and development of the PGD organism.

Materials and Methods

Experiment 1

One hundred and twenty channel catfish fingerlings (8.0 g) were acclimated for 24 h in 75 l aquaria at 21°C. The treatment groups (n= 10/tank) were as follows: *H. exilis* injected + *A. ictaluri* challenge (H+A+); *H. exilis* injected + no challenge (H+A-); *A. ictaluri* injected + *A. ictaluri* challenge (A+A+); *A. ictaluri* injected + no challenge (A+A-); positive and negative controls. There were 2 replicates/treatment. Positive fish were *A. ictaluri* challenged and negative fish were not challenged. One week after challenge all fish were killed with MS-222 (tricaine methanesulfonate), necropsied and all organs were collected and placed in buffered 10% neutral formalin until further processing.

Henneguya exilis spores were collected from cysts in gills of infected catfish, and *A. ictaluri* spores were obtained from *D. digitata* worms collected from ponds containing a catfish population with confirmed PGD (Pote et al., 1994). *Henneguya exilis* spores (5×10^5/ml) or *A. ictaluri* spores (1×10^4/ml) were sonicated and 0.2 ml doses

of each was injected into the peritoneal cavity of 40 fingerlings/ myxozoan. Fingerlings were moved to 19 l aquaria (10 fish/aquarium). One week after intraperitoneal injection of *H. exilis* or *A. ictaluri* sonicated solutions, water was lowered (7.5 cm deep) in the tanks and 15,000 fresh *A. ictaluri* spores were added to the tanks where fish were to be challenged .

Experiment 2

One hundred and seventy catfish fingerlings (8.0 g) were acclimated in 75 l aquaria at 21°C for 24 hours. After 24 hours one hundred and thirty received an intramuscular injection of dexamethasone (Dx) (Beecham Laboratories) in sterile physiologic saline solution at 0.55 mg/kg of body weight as an immunosuppressive treatment. After 1 week 70 dexamethasone injected (Dx1+) and 40 non-injected (Dx1-) fish were removed from the 75 l aquaria and placed in eleven 19 l tanks (n= 10/tank). The water level was lowered (7.5 cm deep) in eight tanks and 15,000 *A. ictaluri* spores were added per tank. After 6 h water levels were raised to previous levels. The treatment groups are noted in Table 6.1.

Table 6.1. Treatment groups for experiment 2.

Treatment group	N=	Dx[a] wk1	Dx[b] wk2	Chall[c] A+	Nec[d] 1wk	Nec[e] 2wk	Nec[f] 3wk
Dx-A+	20	-[g]	-	+[h]	+	-	-
Dx-A-/1	6	-	-	-	+	-	-
Dx-A-/2	6	-	-	-	-	+	-
Dx-A-/3	8	-	-	-	-	-	+
Dx+A-	10	+	-	-	+	-	-
Dx1+A+/1	20	+	-	+	+	-	-
Dx1+A+/2	20	+	-	+	-	+	-
Dx1+A+/3	20	+	-	+	-	-	+
Dx2+A+/1	20	-	+	+	+	-	-
Dx2+A+/2	20	-	+	+	-	+	-
Dx2+A+/3	20	-	+	+	-	-	+

[a] Dexamethasone -followed by 1 week (wk) before challenge
[b] Dexamethasone -followed by 2 wk before challenge
[c] Challenge with *A. ictaluri* (15,000 spores/tank)
[d] Necropsy 1 wk post challenge
[e] Necropsy 2 wk post challenge
[f] Necropsy 3 wk post challenge
[g] -= not done
[h] += done

Histology and indirect fluorescent antibody test (IFAT)

All fish in experiments 1 and 2 were euthanized with a lethal dose of MS-222, the tail was severed and blood was collected using 1.5 ml microcentrifuge tubes. Blood samples from fish in the same tank (n= 10 fish/tank) were pooled. After holding the blood for 6 h in the refrigerator at 4°C it was centrifuged at 300 x g and 10°C for 5 min and sera were collected and stored at -20°C until use. Fish were necropsied, all organs (gills, stomach, intestines, head and trunk kidneys, liver, spleen, heart, skin, muscle, and brain) were collected and placed in 10% formalin for at least 48 hours before processing. For indirect fluorescent antibody test (IFAT) a technique described in the previous chapter was used. Briefly 100 *A. ictaluri* spores were air-dried on glass microscope slides, fixed in cold acetone for 15 min, and incubated for 30 min at 37°C with the test sera. After washing, monoclonal antibody against catfish immunoglobulins (from Dr. Norman Miller, University Medical Center, Jackson, MS provided to Dr. J. Ainsworth, College of Veterinary Medicine, Mississippi State University) was incubated again for 30 min at 37°C. Specific antibodies were detected using goat fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulins (Sigma, St. Louis, MO). Washes were done with phosphate buffered saline (0.01 M phosphate and 0.4 M sodium chloride PBS) pH 8.0. Coverslips were mounted using Vectashield mounting media

(Vector Laboratories, Burlingame, CA). Nonimmunized catfish and mouse sera were used as negative controls while serum from a mouse immunized against *A. ictaluri* was used as a positive control. *Aurantiactinomyxon ictaluri* spores were examined using a Zeiss standard microscope (Carl Zeiss, Oberkochen, West Germany) with an epi-fluorescence condenser and a 100 W xenon arc lamp.

Statistical analysis

Data were analyzed using the chi-square test of independence followed by confidence intervals (CI) to compare groups with respect to the proportions of lesions and organisms detected in non-gill organs. All tests were performed at the 0.05 level of significance and 95% CI on differences between proportions were calculated. Computations were done using the Statistical Analysis System (SAS, 1987).

Results

Experiment 1

One week post challenge with *A. ictaluri* spores, 100% of the positive control fish had inflammatory lesions and myxozoan organisms characterisitic of PGD in the gills (Figure 6.2A). Lesions and organisms were also detected in 30% of the fish in the head and trunk kidneys, liver, and spleen (Table 6.2). Most organisms in non-gill organs appeared to be degenerated (Figure 6.1). Fish injected

with antigenic solutions of *H. exilis* or *A. ictaluri* and challenged one week later with spores of *A. ictaluri* (H+A+ and A+A+) all were positive for lesions and organisms in their gills similar to those observed in the positive control group (Table 6.2). However non-gill organs with lesions and organisms were detected in 45% of the H+A+ and 21% of the A+A+ fish. No statistical differences were found among the proportions of H+A+ (45%), A+A+ (21%), and positive control (30%) fish with lesions and organisms in non-gill organs using the chi-square test; but large values found for the 95% CI for the differences among the proportions suggested that biologically significant differences might have been detected had fish numbers been increased. Proliferative gill disease organisms in non-gill locations of A+A+ fish were similar in appearance to those in the positive control group, but in the H+A+ the organisms did not show any sign of cell death after one week of infection (Figure 6.2). Negative controls, and fish injected with *H. exilis* or *A. ictaluri* but not infected (H+A- and A+A-) did not have lesions or PGD organisms in any organ or tissue examined. Pooled sera samples from all groups of fish in this study did not react with *A. ictaluri* spores by IFAT.

Experiment 2

At 7 days post-infection all positive control fish (Dx-A+) had inflammatory lesions and PGD organisms in gills and

60% of these fish had organisms or lesions in non-gill organs (Table 6.3). Non-gill organs containing organisms were the head and trunk kidneys, liver, spleen, and heart. Negative control fish treated (Dx+A-) and untreated (Dx-A-) were all negative for lesions or organisms. After *A. ictaluri* challenge at one week (Dx1+A+) and 2 weeks (Dx2+A+) in the Dx-treated fish, histological sections showed lesions and organisms in 100% of gills as in the positive control group. However, 47.4% of the Dx1+A+ and 95% of the Dx2+A+ fish showed lesions and organisms in non-gill sites as compared to 60% for positive controls (Table 6.3). The proportions of Dx2+A+ fish (95%) was significantly bigger ($p < 0.05$) than those of Dx1+A+ (47.4%) and positive control fish (60%) with lesions and organisms in non-gill organs. Calculated 95% CI for the differences between the proportion of Dx1+A+ (47.4%) and positive control (60%) fish with lesions and organisms in non-gill organs showed large values suggesting that differences may be detected with larger fish numbers.

Fourteen days after *A. ictaluri* challenge, the Dx2+A+ fish were the only group that had characteristic PGD lesions with 60% of the fish having lesions and 35% having myxozoans in gills. Non-gill lesions and organisms were detected in 5% (n=1) in the same group (Table 6.3). In this single fish the observed PGD organisms outside the gill appeared degenerative (Figure 6.1). In both Dx1+A+ and Dx2+A+ fish

PGD organisms were observed in unicellular and multicellular forms and the organisms were surrounded by varying degrees of inflammatory reactions (Figure 6.3). However, the parasitic stages in the Dx treated fish did not appear to develop any further than those stages observed in the control fish (Figure 6.2). No lesions or organisms were noted in any treatment groups at 21 days post-challenge with *A. ictaluri*.

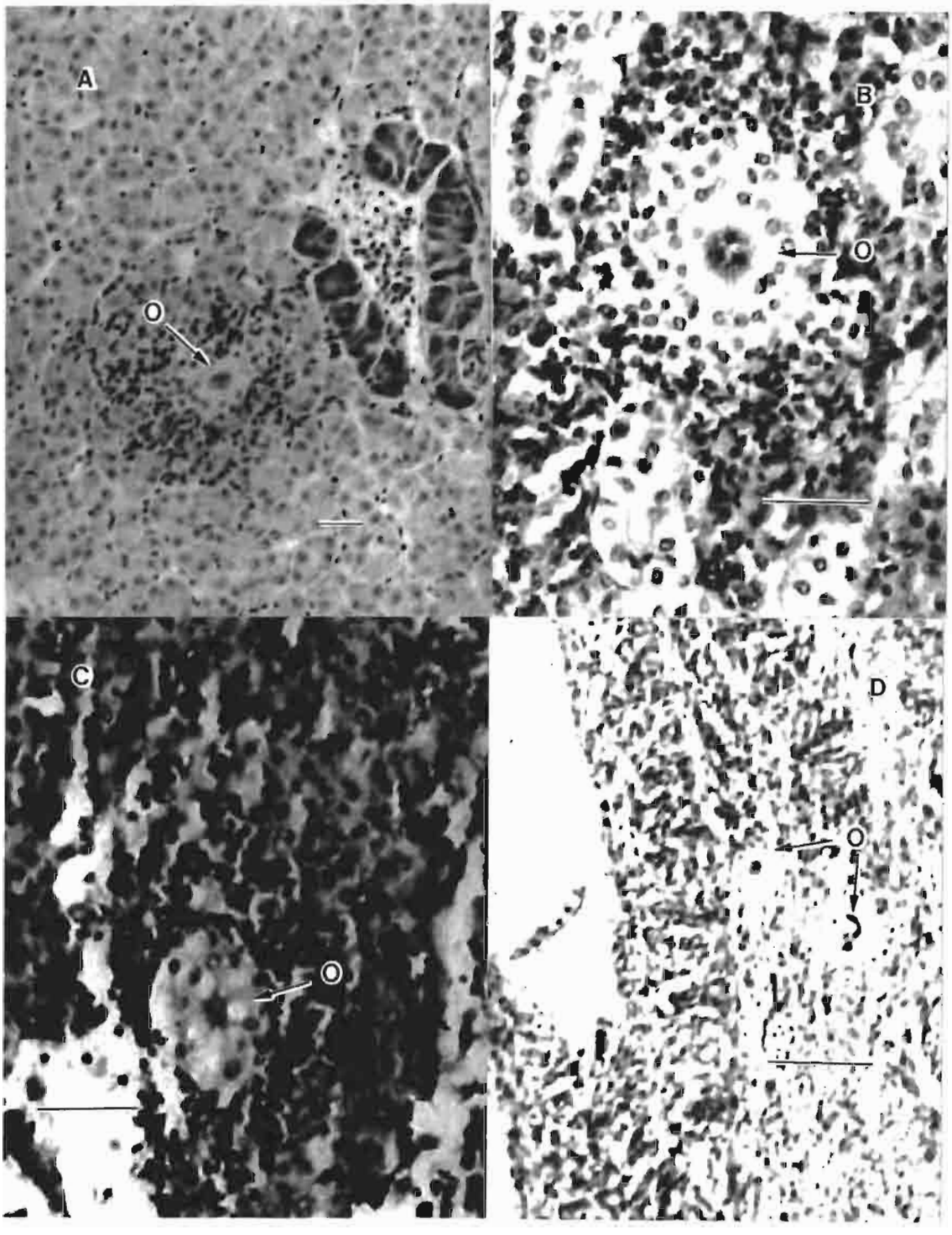

Figure 6.1. Photomicrographs of proliferative gill disease organisms in a degenerating stage in tissue sections of (A) liver, (B) trunk and (C) head kidneys, and (D) gills from channel catfish. O=organism. Bars=10μm

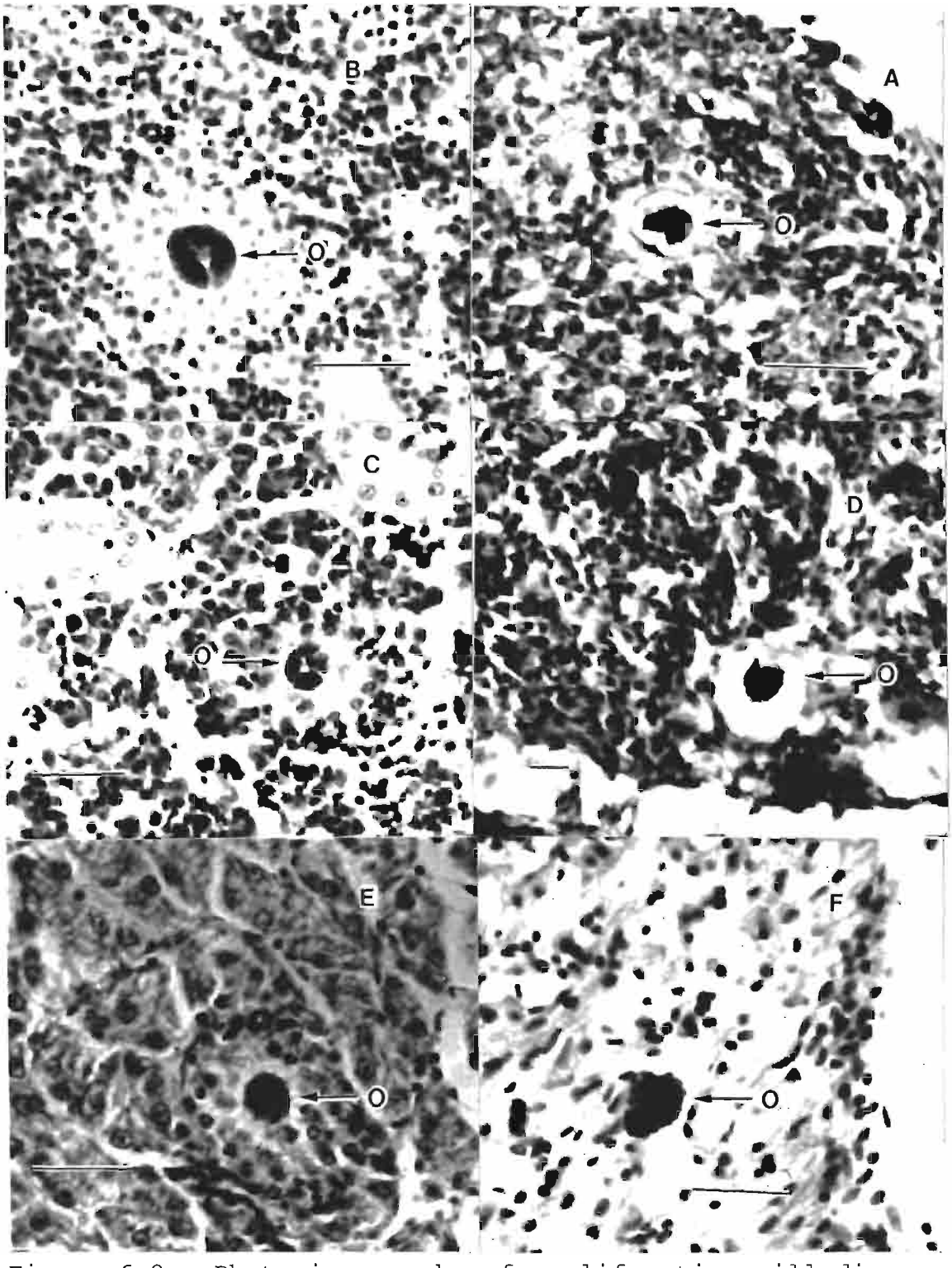

Figure 6.2. Photomicrographs of proliferative gill disease organisms in tissue sections of (A) gill, (B) head and (C) trunk kidneys, (D) spleen, (E) liver, and (F) heart from channel catfish. O=organism. Bars=10μm

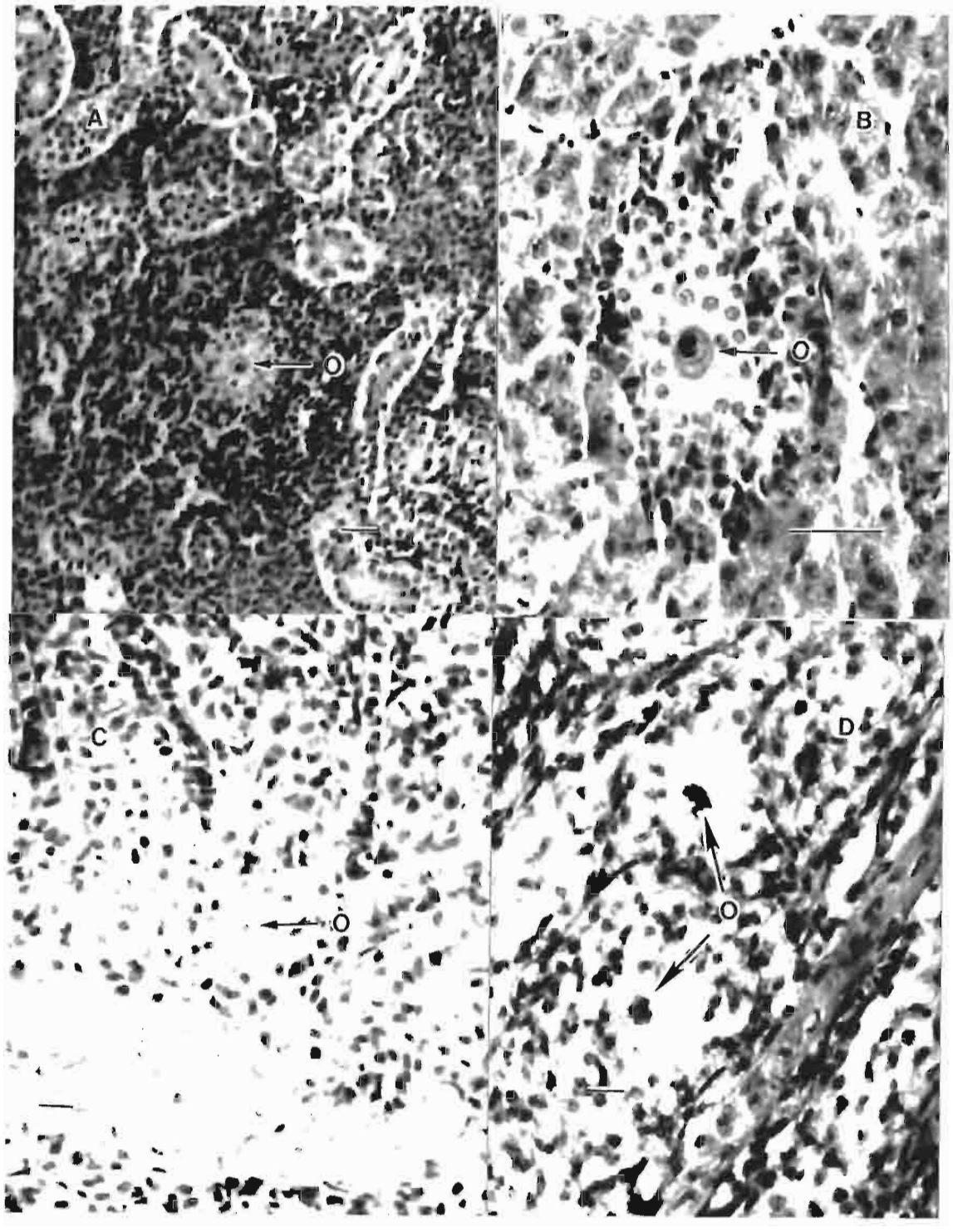

Figure 6.3. Photomicrographs of proliferative gill disease organisms in unicellular forms in tissue sections of (A) trunk kidney, (B)liver, and (C) gill, or without inflammatory reaction in (D) the gill. O=organism. Bars=10μm

Discussion

Lesions and developmental stages of PGD organisms observed in these experiments were similar to those previously described in artificial infections (Bowser et al., 1985; Duhamel et al., 1986; MacMillan et al., 1989; Burtle et al., 1991; Styer et al., 1991) or wild channel catfish (Thiyagarajah, 1993). In the present experiments, infected fingerlings had severe granulomatous branchitis. Gill filaments were focally or diffusely thickened by a mixed cellular infiltrate and they showed fused gill lamellae with epithelial hypertrophy and hyperplasia. Non-gill organs showed mild inflammatory lesions consisting of a few macrophages and lymphocytes surrounding the plasmodia of the PGD organisms.

In experiment 1 fish injected with *H. exilis* and *A. ictaluri* prior to being challenged with *A. ictaluri* (H+A+ and A+A+) had lesions and organisms in the gills of all experimental fish similar to those in the gills of the positive controls. No statistical differences were found among the proportions of H+A+ (45%), A+A+ (21%), and positive control (30%) fish with lesions and organisms in non-gill organs using the chi-square test; but large values found for the 95% CI for the differences among the proportions suggested that biologically significant differences might have been detected had a larger number of fish been used. Lesions and organisms in non-gill organs

were observed more frequently in H+A+ fish (45%) than in the positive control fish (30%). Furthermore most PGD organisms in non-gill organs of H+A+ fish did not show necrosis and had fewer inflammatory cells surrounding organisms (Figure 6.2B to E). MacMillan et al. (1989) proposed that occurrence of PGD organisms in non-gill organs without extensive host response suggested that either immunosuppression or antigenic mimicry occurred in these fish. In the case of the H+A+ fish, immunosuppression may have occurred. More interestingly the A+A+ fish showed lesions and organisms less frequently (21%) in non-gill organs than the positive control fish (30%). Despite the fact that lesions and organisms were similar to those observed in the positive fish, there was either a decrease or a delay in their development in the non-gill organs since fewer fish were affected at 7 days post infection.

Future studies need to examine the amount of antigenic material injected or numbers of *A. ictaluri* spores used for an infection. Also, fish should be maintained for more than one week post challenge to track developing myxosporean stages.

In experiment 2 the effects of dexamethasone on the development of PGD organisms in fish was demonstrated by the frequency of occurrence and the level of development of the lesions and organisms in non-gill organs. The drug appeared to be more effective when injected 2 weeks before challenge

with *A. ictaluri* (Dx2+A+). This would explain the significantly higher ($p < 0.05$) frequency of lesions and organisms in the Dx2+A+ (95%) fish than in the Dx1+A+ (47%). The frequency of lesions and organisms in non-gill organs was higher in the positive control fish (60%) than in the Dx1+A+ fish (47.4%) which may indicate that a one week post-treatment with Dx was not sufficient to suppress the immune system. However, the proportion of Dx1+A+ fish (47.4%) was not significantly different ($p > 0.05$) from the positive control (60%) fish with lesions and organisms in non-gill organs; but calculated 95% CI for the differences between the two proportions showed large values suggesting that differences may have been detected with larger fish numbers. Some PGD organisms observed in gills as well as in non-gill organs of Dx2+A+ fish remained in unicellular forms even one week post infection (Figure 6.3A to C). The parasitic stages in both Dx1+A+ and Dx2+A+ fish did not undergo more development than those observed in the control fish (Figure 6.2). No developmental stages of PGD organisms were seen in this study 21 days post infection which was contrary to the results of MacMillan et al. (1989). The infection model in this study used pure *A. ictaluri* administered once whereas MacMillan et al. (1989) used PGD positive mud with fish exposed continuously during the entire period of the experiment. No lesions or organisms were observed in Dx-A+ and Dx1+A+ fish 2 weeks after *A. ictaluri* challenge. No

organisms or lesions were observed in the Dx2+A+ fish at 21 days post infection. The dexamethasone treatment in the Dx2+A+ fish not only increased the number of fish with lesions and organisms in non-gill organs (95%) but also maintained those lesions and organisms longer in the fish when compared to the positive control (60%). More importantly some of the PGD organisms either were at the unicellular stage or were not contained in a cyst as was observed in the positive control fish (see Fig 6.3). Furthermore inflammatory lesions associated with organisms in this group appeared to be less intense in all organs.

The number of *A. ictaluri* spores used for the infections were 7.5x higher than those usually used in this laboratory, around 2,000 spores/10 fish(Pote et al., 1992); nevertheless, no mortality was observed for both experiments. Interestingly, no antibody specific for *A. ictaluri* was detected in pooled sera collected from fish of all experimental groups using IFAT.

Table 6.2. Percentages of infection of channel catfish fingerlings by *Aurantiactinomyxon ictaluri* after injection of either sonicated *Henneguya exilis* or *A. ictaluri* spores.

Treatment	%Fish with lesions in gills	%Fish with organisms in gills	%Fish with lesions and organisms in non-gill organs
Negative control	0	0	0
Positive control	100	100	30[a]
H+A+[1]	100	100	45[a]
H+A-[2]	0	0	0
A+A+[3]	100	100	21[a]
A+A-[4]	0	0	0

[1]*H. exilis* injected with challenge by *A. ictaluri*
[2]*H. exilis* injected without challenge
[3]*A. ictaluri* injected with challenge by *A. ictaluri*
[4]*A. ictaluri* injected without challenge

[a]All 3 proportions of fish followed by similar lowercase superscript are not significantly different ($p > 0.05$) by the chi-square test. However the results of 95% confidence intervals following the chi-square test have suggested that biologically significant difference might have been found with greater fish numbers per treatment group.

Table 6.3. Percentages of infection of channel catfish fingerlings by *Aurantiactinomyxon ictaluri* after immunosuppression with dexamethasone (Dx).

Treatments	Days after challenge with *Aurantiactinomyxon ictaluri*					
	7 Days			14 Days		
	%Fish with gill L[1]	%Fish with gill O[2]	%Fish with non-gill L and O	%Fish with gill L	%Fish with gill O	%Fish with non-gill L and O
Dx+A-[3]	0	0	0	-	-	-
Dx-A-[4]	0	0	0	0	0	0
Dx-A+[5]	100	100	60[a]	-	-	-
Dx1+A+[6]	100	100	47.4[a]	-	-	-
Dx1+A+	-	-	-	0	0	0
Dx2+A+[7]	100	100	95[b]	-	-	-
Dx2+A+	-	-	-	60	35	5

[1]L= Lesions
[2]O= Organisms
[3]Dx+ = Dx treated fish; A- = nonchallenge with *A. ictaluri*
[4]Dx- = Dx untreated fish
[5]A+ = Challenge with *A. ictaluri*
[6]Dx1+ = Dx treated 1 week prior to challenge
[7]Dx2+ = Dx treated 2 weeks prior to challenge

[ab] Percentages followed by different lowercase superscript are significantly different ($p < 0.05$) by the chi-square test; 95% confidence intervals following the chi-square test have suggested that biologically significant difference might have been found between the percentages of Dx1+A+ fish (47.4%) and the positive controls (60%) had greater fish numbers been used.

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CHAPTER VII

SUMMARY AND CONCLUSIONS

Monoclonal and polyclonal antibodies against *Henneguya exilis* and polyclonal antibodies against *Aurantiactinomyxon ictaluri* were produced. This study failed to produce monoclonal antibodies against *A. ictaluri*. This may have been due to the low number of spores used for mice immunizations. Other studies which have produced monoclonal antibodies to myxozoans have used at least 1 x 10^6 spores/ dose of immunization (Bartholomew et al, 1989) . Due to the difficulties in obtaining *A. ictaluri* spores, only 2 x 10^4 spores/ dose were used to attempt to produce monoclonal antibodies to *A. ictaluri*. Polyclonal sera were adsorbed in live catfish to reduce background staining in immunoperoxidase and indirect fluorescent antibody tests (IFAT). Using polyacrylamide gel electrophoresis and Western blot analysis, all monoclonal antibodies to *H. exilis* tested reacted to a 117 kD component of pure *H. exilis* spore antigen. Difference in number of bands in Western blots between adsorbed and non-adsorbed polyclonal sera against *H. exilis* suggested that the parasite may mimic host antigens.

In cross-reactivity studies using IFAT, the *H. exilis* monoclonal antibody, tested against nine different myxosporeans and actinosporeans, reacted only to *Henneguya* sp. spores either in pure form or in infected tissue sections. All adsorbed polyclonal sera to *H. exilis* and *A. ictaluri* reacted with all myxozoans with the exception of the polyclonal serum against *H. exilis* which did not react to PGD organisms in infected catfish tissues. This suggested the presence of stage-specific antigenic determinants for the gill stage of the PGD organism.

Specific-pathogen-free (SPF) catfish fingerlings were experimentally infected with *A. ictaluri* and were analyzed by immunocytochemistry (IFAT) using the adsorbed polyclonal serum against *A. ictaluri*. At 24 hours post infection fluorescent intracellular forms of the PGD organisms were observed in skin, buccal cavity, and stomach which suggested that these sites may represent portals of entry for *A. ictaluri* into the fish. Unicellular stages of this organism were also found in heart and liver blood vessels. By 96 hours post-infection, the organisms could not be detected in fish tissues with the exception of the typical multicellular trophozoites remaining in the gills. Early detection of PGD organisms using IFAT could help in prevention of PGD from catfish ponds.

Differences in the frequencies of lesions and PGD organisms in non-gill organs were found in SPF catfish

injected with sonicated *H. exilis*, *A. ictaluri* or dexamethasone prior to being challenged with live *A. ictaluri*. Frequencies of infections in those fish immunized with *H. exilis* (45% infected), *A. ictaluri* (21% infected), and positive control fish (30% infected) suggested that the *H. exilis* immunization might have created some immunosuppression while *A. ictaluri* immunization may have protected the fish from subsequent challenges with *A. ictaluri*. In catfish injected with dexamethasone prior to challenge, life cycle stages of PGD organisms were associated with less inflammatory reaction when compared to the positive control. No antibody against *A. ictaluri* was detected by IFAT in pooled sera collected from all groups of catfish. Further research needs to be done to complete the study of the life cycles of myxozoans and characterize the nature of the fish defense mechanisms against mysxosporeans.