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Portals of entry and systemic localization of proliferative gill disease organisms in channel catfish *Ictalurus punctatus*

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ABSTRACT: Immunized rabbit serum adsorbed in live catfish was used in indirect fluorescent antibody test (IFAT) to detect developmental life stages of *Henneguya ictaluri* n. sp. This myxozoan parasite is associated with proliferative gill disease in channel catfish *Ictalurus punctatus* (Rafinesque) in the USA. Specific pathogen free fingerlings were experimentally infected with the actinosporean stage of *H. ictaluri* and necropsied 24, 48, 72, and 96 h post-infection. At 24 h post-infection parasite stages were observed primarily in the gastric mucosa and submucosa but were also observed in the skin and buccal cavity. Ovoid organisms were detected in heart and blood vessels of the liver. From 48 to 72 h after exposure, fewer fluorescent organisms were located in all organs, with the exception of the gills, than were observed at 24 h. These organisms appeared to be degenerating except for those in the gills, which appeared to be multinucleated. By 96 h post-infection, the organisms could not be detected in fish tissues with the exception of the stages in the gills, which appeared to be a preferred site of development. Throughout the entire 96 h period of study, no stage 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.

KEY WORDS: Proliferative gill disease · PGD · *Aurantiactinomyxon ictaluri* · *Henneguya ictaluri* n. sp. · Myxozoa · Actinosporean · Myxosporean · Polyclonal antibodies

INTRODUCTION

Proliferative gill disease (PGD), or hamburger gill disease, is associated with high mortalities in commercial channel catfish *Ictalurus punctatus* (Rafinesque) in the USA. The disease is characterized by branchial inflammation, epithelial hyperplasia, lysis of filamentous cartilages, lamellar fusion, and the presence of myxozoan trophozoites usually in the gills (MacMillan et al. 1989, Burtle et al. 1991, Bellerud et al. 1995).

Several myxosporeans including *Henneguya* sp. (Bowser et al. 1985), *Sphaerospora* sp. (Hedrick et al. 1990), and *Aurantiactinomyxon* sp. (Burtle et al. 1991, Styer et al. 1991, Pote et al. 1992) had been previously implicated as etiologic agents for PGD. It has since been shown that the life cycle of the PGD organism is similar to other myxozoan life cycles previously described such as that of *Myxobolus cerebralis* (Markiw & Wolf 1983, Wolf & Markiw 1984), *Hoferellus carassii* (El-Matbouli et al. 1992), *Myxobolus arcticus* (Kent et al. 1993), *Hoferellus carassii* (Yokoyama et al. 1993), and *Ceratomyxa shasta* (Bartholomew et al. 1997), which linked an actinosporean in an aquatic oligochaete with a myxosporean in a fish species. Molecular data have further confirmed that the actinosporean stage and the myxosporean counterparts of *M. cerebralis* (Andree et al. 1997), *C. shasta* (Bartholomew et al. 1997), *Henneguya exilis* (Lin et al. 1999), and *Tetracapsula bryosalmonae*, syn. *Tetracapsula renicola* n. sp. (Kent et al. 2000), are life stages of single organisms

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(Canning et al. 2000, Okamura et al. 2001). Similarly with PGD, when catfish were exposed to the actinosporean stage, *Aurantiactinomyxon* spp., isolated from the aquatic oligochaete *Dero digitata* (Burtle et al. 1991, Styer et al. 1991), PGD organisms were observed in the gills. Based on recent molecular data, it was further shown that the actinosporean stage, *A. ictaluri*, the PGD myxozoan stages present in the gills, and a subsequent myxosporean gill stage, *Henneguya ictaluri* n. sp., had identical rRNA gene sequences, thus confirming that the actinosporean, *A. ictaluri*, is a life stage of the myxosporean, *H. ictaluri* n. sp. (Pote et al. 2000, Hanson et al. 2001)

Although the source of infection causing PGD has been confirmed, the route of infection of *Henneguya ictaluri* is not clearly understood. Pote & Waterstrat (1993) showed that the actinosporean *Aurantiactinomyxon* sp. produced a motile amoeba-like stage upon its exposure to catfish gill filaments. However, in addition to the gills, PGD trophozoites have been shown in liver, head and trunk kidneys, spleen, and brain of naturally infected catfish (MacMillan et al. 1989, Bellerud 1993). El-Matbouli et al. (1999) showed using the triactinomyxon spores of *Myxobolus cerebralis* that portals of entry into the fish were the secretory openings of the mucous cells in the epidermis, buccal cavity, and gills of rainbow trout. Light and electron microscopy showed the proliferation of this parasite in the epidermis and central nervous system of trout (El-Matbouli et al. 1995). While much is known about the route and course of infection with *M. cerebralis* in trout, very little is known about the point of entry of the actinosporean stage of *H. ictaluri*, the morphology of the early stages in the fish, or the mechanism by which this organism is transported systemically. The purpose of this research was to determine the point of entry of the actinosporean stage of *H. ictaluri* into the catfish and detect the early life stages of this parasite and its dissemination in the channel catfish.

MATERIALS AND METHODS

Preparation of spores and infection of catfish. Spores of the actinosporean stage of *Henneguya ictaluri* 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 aperture) to remove debris. A dissecting microscope was used to examine material captured on the screen and isolate *Dero digitata*. Using a modified technique (Yokoyama et al. 1991), worms were placed in sterile water, rinsed with 3 exchanges of sterile water, individually transferred to wells of 96-well plates, and covered with water. The plates were incubated at room temperature until actinosporean spores were released by the infected worms. Those actinosporean spore stages of *H. ictaluri*, *Aurantiactinomyxon* spp. (Marques 1984, Bellerud 1993, Pote et al. 2000) were concentrated into conical 15 ml polystyrene test tubes and counted.

Specific pathogen free (SPF) channel catfish fingerlings were placed in three 19 l flow through tanks. Twenty fingerlings each were placed in Tanks 1 and 2, and 10 in Tank 3. Before infection the water was lowered in all tanks to a depth of approximately 7.5 cm and *Henneguya ictaluri* actinosporean spores were added to Tanks 1 and 2 (2000 spores $tank^{-1}$); Tank 3 served as a non-infected control. Six hours later, the water level was raised to fill all tanks and flow through water was continued. At 24, 48, 72, and 96 h post-infection, fish (n = 5 each) were collected from Tanks 1 and 2, and a single fish from Tank 3. All fish were necropsied, and tissues (gills, stomach, intestines, head and trunk kidneys, liver, spleen, heart, skin, muscle, and brain) were collected and placed in neutral buffered 10% formalin for at least 48 h before routine histopathology processing. Paraffin-embedded histological sections of all tissues (4 µm thick) were either stained with Mayer's hematoxylin and eosin (H&E) and examined by light microscopy (100 or 400 × magnification) or deparaffinized, rehydrated, rinsed in distilled water, and used in immunocytochemistry.

Production of polyclonal antiserum and its use in the indirect fluorescent antibody test. Clean spores of the actinosporean stage of *Henneguya ictaluri* were collected from *Dero digitata* (Pote et al. 1994) and adjusted to a concentration of 1×10^5 spores ml^{-1}. Polyclonal antibodies to the actinosporean stage of *H. ictaluri* were made as described previously (Belem 1994). Briefly, concentrated *H. ictaluri* spores were sonicated and used as an antigen to immunize a rabbit. The antigen was diluted with sterile distilled water to approximate the delivery of 2×10^4 spores of *H. ictaluri* $dose^{-1}$. Immune and non-immune rabbit sera were adsorbed in live catfish according to a procedure previously described by Belem (1994). Adsorbed and non-adsorbed sera, and non-injected SPF catfish serum were all tested for specificity by indirect fluorescent antibody test (IFAT) on pure *Aurantiactinomyxon ictaluri* spores and on histopathologically confirmed PGD-infected gill sections.

Polyclonal antibodies were incubated for 30 min at 37°C with air-dried actinosporean spores of *Henneguya ictaluri* or tissue sections on glass microscope slides, and then fixed in cold acetone for 15 min. Paraffin-embedded tissue sections were deparaffinized, rehydrated, and rinsed in distilled water before use (Haines & Chelack 1991). Specific antibodies were de-

tected by using goat fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulins (Sigma, St. Louis, MO). Washes were done with phosphate-buffered saline (PBS) (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 and photographed using a Zeiss standard microscope (Carl Zeiss, Oberkochen, West Germany) equipped with an epi-fluorescence condenser and a 100 W xenon arc lamp.

RESULTS

Small parasite stages were observed in the mucosa and submucosa of the stomach using IFAT at 24 h post-exposure to the actinosporean spores of *Henneguya ictaluri* (Fig. 1). Organisms were also seen in the epithelia of the skin and buccal cavity. Ovoid stages were visible in the heart and blood vessels of the liver (Fig. 2). All the gills examined demonstrated the presence of these stages (Fig. 3). Small fluorescent 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.

From 48 to 72 h after exposure, stages appeared in the gastric glands of the stomach. Fewer stages were located in the skin and the mouth than were observed at 24 h. Heart and liver blood vessels also showed fewer ovoid forms than were seen at 24 h. The larger stages appeared to decrease in the gills with time but the remaining organisms appeared to be larger or degenerating and were located in gill filament stroma along the cartilage. At 72 h, most gills showed very bright fluorescent forms without obvious nuclei. Inclusions in hematopoietic cells of the kidneys and spleen parenchyma were less numerous and the fluorescent intensity had decreased compared with organisms seen at 24 h.

At 96 h post-exposure, most of the fluorescent organisms had disappeared from the stomach, heart, liver, spleen, and kidneys and only the stages in the stroma of gill filaments were present (Fig. 4). Furthermore, this stage was 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 results of tissue sections of all organs and tissues stained with H&E were negative except for the gills. An organism was first seen at 48 h after exposure in a single case; the organism appeared to be uninucleated and was located in the stroma of gill filaments. Multinucleated trophozoites were found in the gills at 72 and 96 h after infection. No organisms were detected with H&E staining or IFAT in any of the negative control fish.

DISCUSSION

This experiment showed that IFAT can be used to detect early stages of PGD organisms in infected channel catfish tissues not normally observed by the more

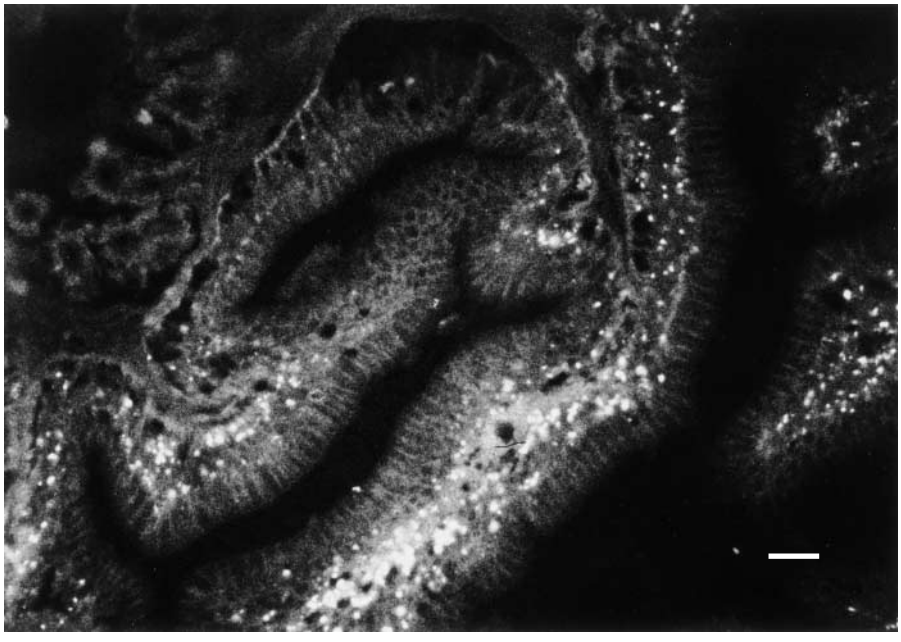

Fig. 1. Fluorescent proliferative gill disease (PGD) organisms in the stomach epithelium of specific pathogen free (SPF) catfish, 24 h post-exposure to the actinosporean stages of *Henneguya ictaluri* n. sp. Scale bar = 10 µm

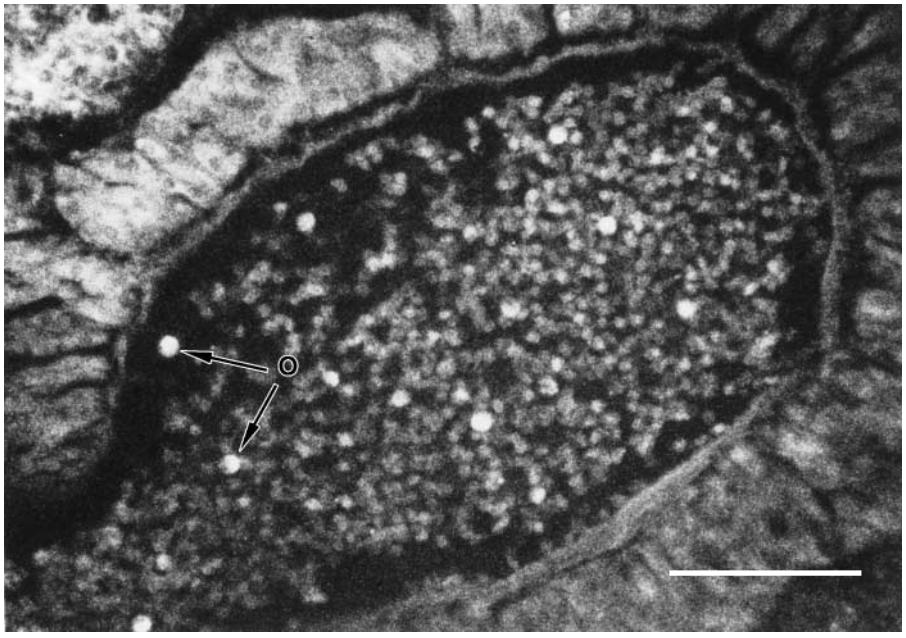

Fig. 2. Fluorescent (PGD) organisms in the liver blood vessel of SPF catfish, 24 h post-exposure to the actinosporean stage of *Henneguya ictaluri*. O = organism. Scale bar = 10 µm

traditional histopathology methods employing H&E staining in conjunction with bright field microscopy. With the use of IFAT, organisms were detected 24 h after infection. Pure actinosporean spores of *Henneguya ictaluri* 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. The 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 before 24 h post-exposure. The present study also corroborated the findings that the actinosporean stage of *H. ictaluri* shed by *Dero digitata* is linked to the PGD organisms observed in catfish gills (Styer et al. 1991, Pote et al. 1992, 2000).

Studies by Pote & Waterstrat (1993) showed the production of a motile actinosporean stage of *Henneguya 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

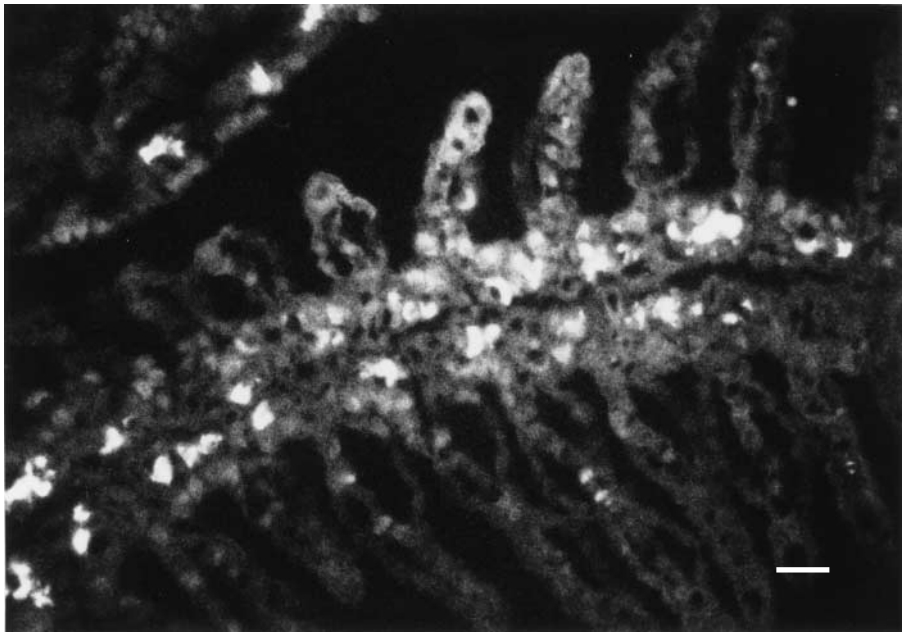

Fig. 3. Fluorescent (PGD) organisms in gills of SPF fish, 24 h post-exposure to the actinosporean stages of *Henneguya ictaluri*. Scale bar = 10 µm

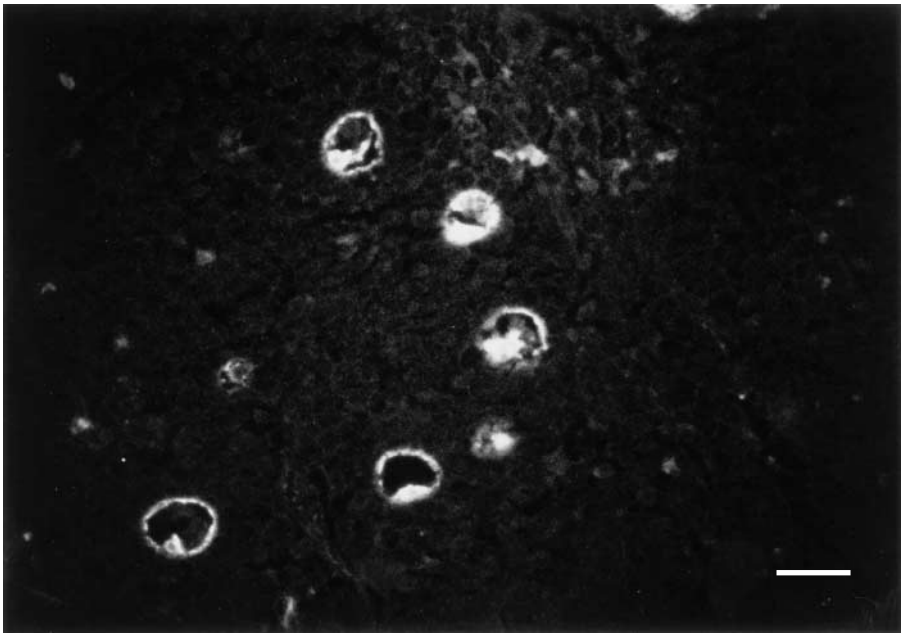

Fig. 4. Fluorescent (PGD) organisms in gills of SPF fish, 96 h post-exposure to the actinosporean stages of *Henneguya ictaluri*. Scale bar = 10 µm

filaments remain unknown, the present study suggests that multiple sites of entry are possible based on the observation of similar fluorescent stages in the skin, the base of the gills, the buccal cavity, and the stomach. This confirmed the work of Yokoyama & Urawa (1997), who also observed the penetration of *Auranti-actinomyxon* sp. into the gills of common carp using fluorescent labeling of actinospores; however, they were not able to show skin penetration with this species. The stomach seemed to be an important entry site 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 & 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 parasite stages in the heart and liver blood vessels in association with red blood cells. Blood circulation could have been responsible for 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 salmonids.

Organisms were degenerating or decreasing in number at 96 h in the stomach, skin, liver, spleen, kidneys, and heart. This indicates 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 shown in traditional histopathology studies (MacMillan et al. 1989). The gills seemed to be preferred sites of development for the PGD myxosporeans since intact multicellular trophozoites were still observed at 96 h post-exposure but had degenerated or were non-existent in other organs.

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