Comparative Medicine
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Vol 52, No 4
August 2002
Pages 354-358

Pseudocapillaria tomentosa, a Nematode Pathogen, and Associated Neoplasms of Zebrafish (*Danio rerio*) Kept in Research Colonies

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Infections with capillarid nematodes were observed in zebrafish (*Danio rerio*) kept at several research facilities and in a large carcinogen exposure study previously conducted at Oregon State University. We report a morphologic description that identifies the worm as *Pseudocapillaria tomentosa*, a common nematode of cyprinid and other fishes. Pathologic lesions associated with the infection ranged from inflammatory changes to aggressive neoplasms of the intestine (i.e., intestinal carcinomas and mixed malignant neoplasms). Capillarid nematodes may have intermediate or paratenic hosts. Using a laboratory transmission study, we confirmed that the parasite has a direct life cycle.

Over the past decade, there has been a tremendous increase in the use of zebrafish as a laboratory model (1), and thus, there has been a concurrent increase in interest concerning diseases that may afflict this fish, particularly in a research setting. Metazoan parasites (helminths and arthropods) are important causes of disease in captive fishes, including warm water species (2, 3). However, because zebrafish colonies usually are kept in aquaria containing dechlorinated city water in recirculating systems, metazoan parasites (particularly those requiring intermediate hosts) are not numerous. Capillarid nematodes infect all classes of vertebrates, and often are pathogenic due to their invasive nature. Several species infect a wide variety of fishes, including aquarium species (4-6). Capillarids are tissue invasive, and thus, some species are highly pathogenic to fishes. Pack and co-workers (7) reported intestinal capillariasis as a cause of wasting disease in zebrafish. We provide a diagnostic service to the zebrafish research community through the Zebrafish International Resource Center (ZIRC; University of Oregon, Eugene, Oreg.). We have received several fish specimens in which this infection was present. We report on the identification of and pathologic changes associated with the infection. The life cycles of capillarids of fishes may require intermediate hosts, such as oligochaete worms (6). However, others may be transmitted directly from fish to fish (8). We also report direct transmission of the capillarid from zebrafish.

Materials and Methods

We conducted an experiment to determine whether the nematode from zebrafish could be transmitted in the absence of intermediate hosts (e.g., oligochaete worms). Infected zebrafish were obtained from a regional aquarium dealer and were kept in flow-through, bare, plastic aquaria at 28°C at Oregon State University (OSU). The water supply was dechlorinated city water. We cohabitated four fish with clinical signs of the infection with 25 uninfected fish (7 weeks old) from our capillarid- free population. These "SPF" recipient fish were derived from the ABC strain that had been reared at the ZIRC for many generations and in which the infection has never been detected. Absence of the infection in these fish is supported by these observations: fish are kept in dechlorinated city water at the ZIRC, all fish enter the facility through a quaratine program in which eggs are disinfected with chlorine at the approximate concentration of 25 ppm for 10 min, and examination of some 100 sentinel fish or fish from diagnostic cases from this facility has never revealed the infection. Recipient fish were transported from the ZIRC to OSU and were kept in flow-through aquaria, again with dechlorinated city water. All fish were fed an artificial, dry diet to eliminate possibilities of contracting the infection through live food vectors.

The exposure aquarium was bare (i.e., no gravel present), and oligochaete worms were not in the system. Fish were kept for five months, and periodically throughout the experiment, wet mount preparations of the intestine of fish with clinical signs of the infection (i.e., emaciation) were examined for presence of the infection. Equal numbers of control fish (unexposed) from the same AB population and kept in the same water system were examined at each sample collection. AB refers to a wildtype zebrafish strain developed by zebrafish researchers (see <http://zfin.org/cgi-bin/webdriver?MIval=aa-wtlist.apg>http://zfin.org/cgi-bin/webdriver?MIval=aa-wtlist.apg). All fish were maintained and treated humanely, and the study was conducted with approval of the Institutional Animal Care Committee (IACC) and the Department of Laboratory Animal Resources at OSU (# 2641).

Fish from our diagnostic service operated by the ZIRC and from a previous 7,12-dimethylbenze[*a*]anthracene (DMBA) exposure study (9) were used for histologic and parasitic morphologic examinations. Infected fish were euthanized by administration

Received: 2/06/02. Revision requested: 4/11/02. Accepted: 5/28/02.
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Supported in part by NIH grant P40 RR12546.
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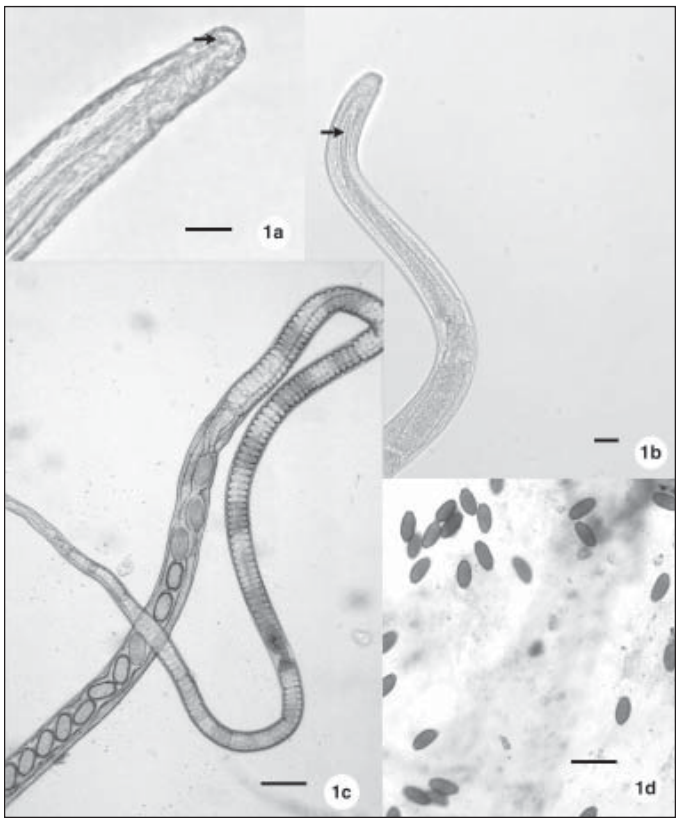

Figure 1. Wet mount preparations of *Pseudocapillaria tomentosa*. (a) Posterior of male with diminutive bursa (arrow). Bar = 25 μm. (b) Posterior of male with smooth spicule (arrow). Bar = 25 μm. (c) Female with distinct stichocysts with alternating light and dark bands. Bar = 100 μm. (d) Numerous eggs observed in wet mount of gut with intestinal adenocarcinoma. Bar = 100 μm.

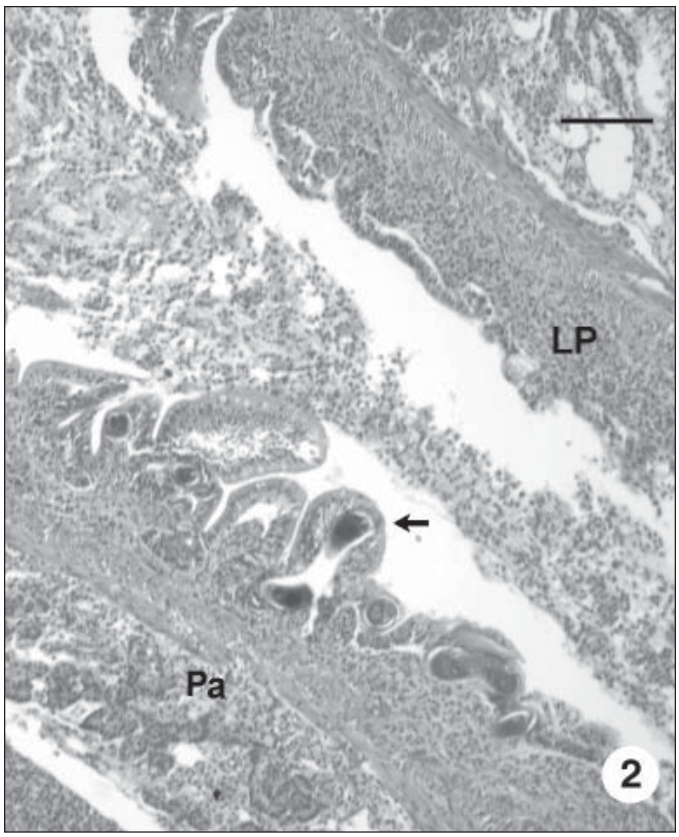

Figure 2. Photomicrograph of a section of a zebrafish with *Pseudocapillaria tomentosa* infection and intestinal neoplasms. Notice diffuse, severe chronic inflammation, extending from the lamina propria (LP) into the visceral cavity. *P* = inflammation in pancreas; arrow = nematode. H&E stain; bar = 100 μm.

of an overdose of MS-222, the abdomen was opened, and the body was preserved in Dietrich's solution. Fish bodies were demineralized, histologic sections (mid-sagittal) of infected individuals were prepared, and slides were stained with hematoxylin and eosin. Wet mount preparations were made of either fresh or 95% ethanol-preserved worms for taxonomic identifications, and measurements were made by use of a digital computer camera system.

Results

Transmission. Periodic examination of the exposed fish revealed the infection throughout the exposure period, whereas all control fish examined at the same sampling times were infection negative. Equal numbers of control fish and exposed fish were examined at each sampling date. For the recipient (exposed) fish, at 10, 16, and 21 weeks after exposure, one of two fish had the infection. At 14 weeks, both fish (2/2) from the exposed group were infected, and at 23 weeks after exposure, four exposed fish were examined and all were test positive for the infection.

Microscopy. The following measurements were from male (n = 5) and female (n = 8) fish (Fig. 1a-c). Length: females, seven to 12 mm; males, four to seven mm. The eggs had distinctive, bipolar plugs, and thin shell walls with minute knobs (Fig. 1d). Eggs (n = 20) measured (mean and range) as follows: width, 30.7 (27 to 35) μm; length, 64.8 (57 to 68) μm. The male bursa was diminutive and indistinct; the posterior end of the male was rounded and lacked caudal alae (Fig. 1a and 1b), and the spicule was smooth (Fig. 1b). Females had stichocysts in alternating dark and light bands, with prominent nuclei (Fig. 1c).

A total of 18% of our diagnostic cases (representing several fish from each case) that we have received since November 2000 were infected with the nematode. This represented specimens from seven research facilities, and a total of 19 of 426 fish (4.5%) that we examined in our diagnostic service. Histologic examination of these infected zebrafish from the diagnostic cases revealed a variety of pathologic changes. In all fish, worms were observed within the epithelium and lamina propria of the intestine (Fig. 2-4). The degree of inflammation associated with the parasite varied widely among individual fish, ranging from mild, locally extensive nonsuppurative inflammation to severe, locally extensive or diffuse, chronic granulomatous, transmural inflammation and associated granulomatous peritonitis. Two fish from these diagnostic cases also had intestinal neoplasms consistent with those subsequently described.

In fish from the DMBA exposure study previously conducted at OSU (9), the nematode infection was observed in only one of 20 groups exposed to DMBA, which represented only one among numerous experiments with a variety of carcinogens in which more than 10,000 fish were examined microscopically. Parasites were observed only in fish exposed to the highest dose of DMBA as fry (i.e., fish exposed to 5 ppm) (9). Interestingly, six fish of

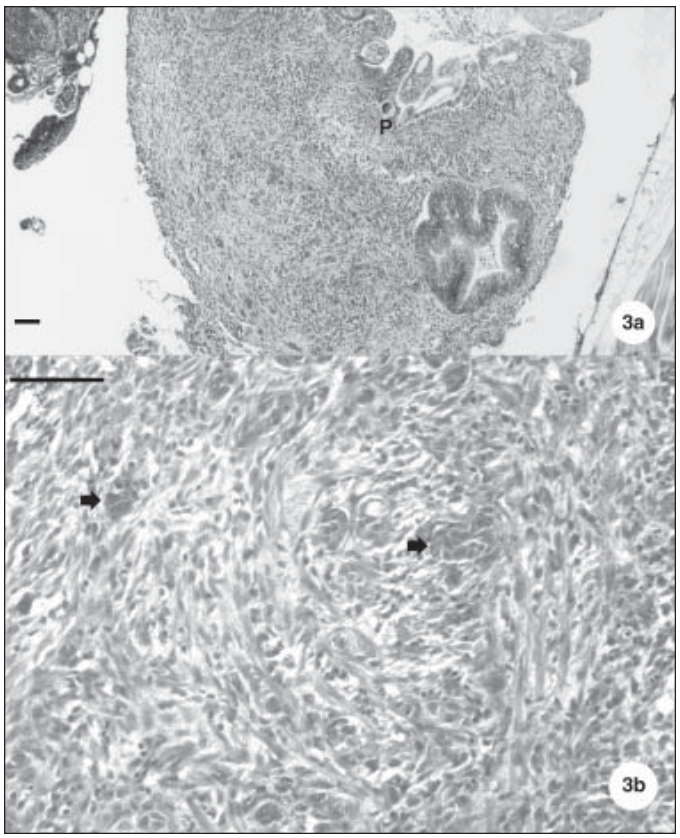

Figure 3. Photomicrograph of a section of a transmural mixed malignant neoplasm of the distal portion of the small intestine. Mass is composed of an admixture of malignant smooth muscle cells and clusters of ascini of malignant enteric epithelium. (a) Profiles of the nematode (P) are evident in the mucosal epithelium, and moderate to severe hyperplasia of the mucosalepithelium is evident in the segment of bowel surrounding the parasite. Increased numbers of lymphocytes and macrophages are evident in the lamina propria and submucosa subjacent to the parasite. (b) Higher magnification reveals clusters of malignant epithelial cells (arrows) admixed with proliferating malignant smooth muscle (spindle) cells. H&E stain; bar = 20 μm.

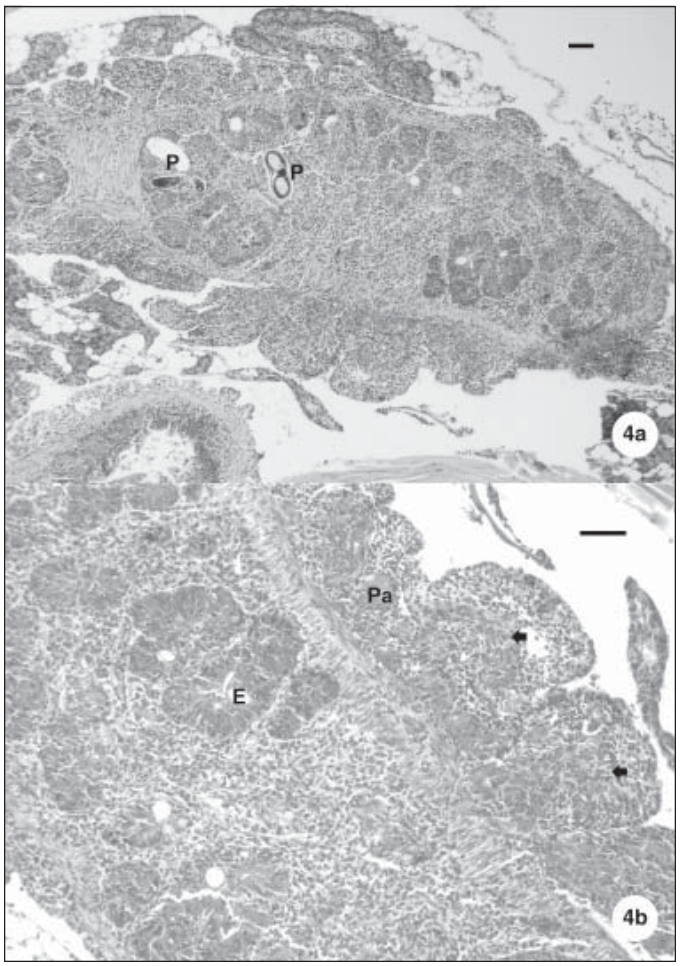

Figure 4. Photomicrograph of a section of an adenocarcinoma of the intestine associated with *Pseudocapillaria* infection. (a) Notice severe diffuse hyperplasia and dysplasia of mucosal epithelium of the intestine associated with intraepithelial profiles of nematodes (P). Moderate diffuse nonsuppurative inflammation is present throughout the mucosa and submucosa, as well as covering the serosal surface. (b) Higher magnification reveals irregular clusters of malignant enterocytes (arrows) admixed with normal exocrine pancreas (Pa) along the serosal surfaces of the intestine. Notice hyperplasia and dysplasia of intestinal epithelium (E) and high mitotic rate in neoplasm. H&E stain; bar = 20 μm.

this group had the infection, and 50% of them had intestinal neoplasms. In contrast, only 13% of the uninfected fish from the same population had these neoplasms. These tumor incidences differed significantly on the basis of χ^2-analysis ($P = 0.03$). The neoplasms from this study and our diagnostic material were usually situated at the intestinal-esophageal junction, and often had nematodes directly associated with the lesions (Fig. 3 and 4). The tumors were locally extensive or multifocal, either intestinal cell carcinomas (Fig. 3) or mixed malignant tumors with epithelial and mesenchymal components (Fig. 4).

Discussion

Measurements and morphologic features of the nematode of this study indicated that they were consistent with those of *Pseudocapillaria tomentosa* (10). Measurements of the females and males in our study were within the range reported by Moravec (4, 10)—females, 7.30 to 12.04 mm long; males, 3.95 to 7.18 mm long. The eggs of our study were comparable, but slightly smaller than those reported by Moravec (4). In that report, eggs were 63 to 78 μm × 30 to 39 μm in diameter. The presence of alternating dark and light coloration of the stichocysts also is a distinguishing characteristic of *P. tomentosa*. The male posterior region was consistent with that of *P. tomentosa* (e.g., sub ventral papillae were present, a pseudobrusa was not observed, and the spicule was not spiny). *Pseudocapillaria tomentosa* (junior synonym *P. brevispicula*) has a broad host specificity, infecting some 25 fishes in the family Cyprinidae and members of other orders, such as Agulliformes (eels), Gadiformes (cod fishes), Salmoniformes (salmon), and Siluriformes (catfishes) (10). *Pseudocapillaria tomentosa* was associated with mortality in captive tiger barbs (*Puntius tetrazona*) (5), and related parasites cause disease in other aquarium fishes. *Capillaria pterophylii* has been recognized for many years as a common pathogen of captive angelfish (*Pterophylum scalare*) and discus fish (*Symphysodon* spp.) (11), and *Capillostrogyloides ancistri* is highly pathogenic to the bushymouth catfish (*Ancistrus dolichopterus*) (12). From laboratory transmission studies, Lomankin and Trofimeko (8) reported that oligochaetes (e.g., *Tubifex tubifex*) can serve as paratenic

hosts for *P. tomentosa*. In that same study, they reported that direct transmission in the absence of worms also is a route of infection. Field studies suggest that oligochaete worms have a relevant role in transmission of the nematode in wild fishes (4). Verification that the *P. tomentosa* can be transmitted in the absence of oligochaete worms indicates that the parasite can proliferate in zebrafish research facilities.

Infected fish often were emaciated, which is consistent with the report by Pack and co-workers (7). Many fish had severe inflammatory changes of the intestine and visceral cavity, and thus, the histologic changes were consistent with the poor health of the fish. The association with the intestinal neoplasms was notable. We have not documented a direct link between the infection and predisposition to the development of this neoplasm, but the links between parasite infections and some neoplasms have been established. Probably the most analogous situation is infection with the nematode *Spirocerca lupi* and esophogeal sarcomas in dogs (13). Thamvavit and co-workers (14) suggested that, in most instances, parasites act as strong promoters, rather than initiators, of neoplasia. This was based on their experiments with *Opisthorchis viverrini* in hamsters and review of other studies. As the neoplasms were observed in diagnostic cases with no known exposure to carcinogens, perhaps there are low amounts of carcinogens in the diets or water at some zebrafish facilities, and the worms act as a promoter in the development of intestinal tumors. This also appears to be the case in the DMBA exposure study (9), where there was a significantly higher prevalence of these tumors in nematode-infected fish than in those without the infection. As we can easily experimentally establish infections of zebrafish with *P. tomentosa*, this may provide an opportunity to elucidate the association between these infections with neoplasia of the gut.

Regarding control of the infection, oligochaete worms may be a source of the infection (although not required in the life cycle) and they should be avoided as a food for zebrafish kept as research animals. Pack and co-workers (7) reported that a mixture of trichlorofon and mebendazole in the form of Fluke-Tabs (Aquarium Products, Glen Burnie, Md.) added to water eliminated the infection. Treated fish gained weight, and when examined later, the infection was not observed. However, untreated controls were apparently not included in the study. Caution should be used when treating fish with mebendazole as it may be embryotoxic and teratogenic, and trichlorofon is a neurotoxic organophosphate (15).

Other anthelminthic drugs have been tested on other fishes infected with nematodes, and may be useful for this infection. Ivermectin is widely used against tissue invading nematodes in veterinary medicine, and has been tested with fish (16, 17). Heckman (18) reported that it controlled nematode infections in sculpins. Ivermectin has been used successfully to treat external copepod infections in salmon, with oral treatment at a dosage of 0.05 mg/kg of body weight, administered twice weekly (19) or with three to six doses spaced three days apart (20). The broad-spectrum anthelmintic levamisole, applied as a bath, has been reported to be effective against the swim bladder nematode *Anguillicola crassus* (21, 22). Lo Wing Yat (23) reviewed treatments for capillarid nematode infections in discus fish (*Symphysodon* sp.). That information was largely from the aquarium fish industry and was not based on rigorous scientific studies. Nevertheless, garlic oil and levamisole in the diet were ineffective or results were extremely variable among fish. Park and co-workers (7) reported that levamisole was not useful for treating *P. tomentosa* in zebrafish, and brood stock zebrafish treated with levamisole have become sterile (24). Given the aforementioned potential problems with antihelminthic compounds in zebrafish, if fish are not highly valuable, the most appropriate choice would be to eliminate the infected population.

In conclusion, *P. tomentosa* has the potential to proliferate and cause appreciable disease in zebrafish colonies. At present, we cannot recommend a specific treatment, and thus, avoidance of infection (e.g., quarantine and screening for the infection) is recommended.

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