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High Mortality Due to *Tetrahymena* sp. Infection in Laboratory-Maintained Zebrafish (*Brachydanio rerio*)

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A large colony of laboratory zebrafish (*Brachydanio rerio*) used in the study of early vertebrate embryogenesis began experiencing acute, unexplained mortality that approached 100% among approximately 30-day-old resident fry. The initial differential diagnosis included ammonia, nitrite, or chlorine toxicosis, as well as iatrogenically induced toxicosis associated with improper sanitation procedures of laboratory equipment. Necropsy of dead and moribund fry prior to fixation revealed swarms of ovoid-shaped, motile, ciliated protozoa with a "spiraling football" motion. Wet mount preparations of various water samples also contained high numbers of similar protozoa. Histologic examination of affected fry revealed numerous, periodic acid-Schiff-positive forms within the body coelom, and epithelial and muscle tissues. The protozoa were consistent morphologically with members of the genus *Tetrahymena*, which is usually a free-living, nonpathogenic ciliated protozoa in fresh and saltwater environments. Relevant disease associated with *Tetrahymena* spp. in viviparous fish has been reported as a result of concurrent disease, immunosuppression, or poor water quality conditions. To the authors' knowledge, this is the first report of an epizootic involving laboratory maintained zebrafish, and the diagnostic course and therapeutic interventions undertaken to alleviate *Tetrahymena* species-associated clinical disease.

The zebrafish, *Brachydanio rerio* (also referred to as *Danio rerio* and the zebra danio), has become an important vertebrate model for development, gene function analysis, and mutagenesis study since the late 1980s. This model is based on the similarity of fundamental molecular mechanisms among vertebrate species. Through creation of mutant phenotypes, the functions of many genes associated with pigmentation, muscular, cardiovascular, and CNS development have been investigated (1, 2). The zebrafish (Hamilton-Buchanan, 1822) is a member of the family Cyprinidae and is an oviparous, sexually dimorphic, freshwater species native to river regions of southern Asia.

Tetrahymena spp. are ciliated protozoa that have worldwide distribution in fresh and salt water. These protozoa are members of the family Tetrahymenidae of the order Hymenostomatida (3). On the basis of recent 18S ribosomal RNA sequencing, *Tetrahymena* spp. appear to be relatives of the more common and well-known tropical aquarium fish pathogen, *Ichthyophthirius multifiliis* (4). *Tetrahymena* spp. are extremely motile, pear- or pyriform-shaped, and measure approximately 30 to 60 × 50 to 100 μm in size. Reproduction is through binary fission that can result in sudden bursts of high numbers of microorganisms when environmental conditions favor replication (5). Occasionally, this microorganism can become parasitic and affect a wide range of hosts. In particular, live-bearing (viviparous) fishes, such as guppies and some cichlids, appear to be most susceptible (6), hence the name "guppy killer disease." Although *Tetrahymena* spp. usually are free-living, non-parasitic ciliates within the biofilms of some water systems, specific strain variability, presence of immunocompromised fish hosts, high stocking densities, and poor water quality (e.g., high organic products) have been implicated as factors influencing the virulence of these microorganisms (6-8). *Tetrahymena* spp. can also be considered secondary invaders of epithelial lesions on fish suffering from concurrent disease due to trauma, bacterial infection, or other parasites (9-11).

Although presence of *Tetrahymena* spp. within a recirculating water system or colonizing the surface epithelium of fish in low numbers is of questionable importance, presence of these microorganisms in large numbers or within the tissues of affected fish suggests pathogenic potential (12). Although the mode of entry into the host has not been well documented, systemic tetrahymenosis usually results in profound disease associated with epithelial necrosis and hemorrhage, myositis, and exophthalmos due to peri-ocular tissue invasion (13). Substantial subdermal and muscle tissue necrosis with minimal inflammatory reaction often is observed (3). Diagnostic evaluation of epizootics usually reveals swarms of these protozoa in environmental and fish wet mount preparations. Microscopic aggregates of parasitic protozoan forms are frequently found in the kidney, brain, gill lamellae, muscle, and subdermal tissue (13). The purpose of the study reported here was to document an epizootic of systemic tetrahymenosis under natural conditions associated with laboratory-maintained zebrafish, the diagnostic course undertaken to investigate the cause of the infection, and the

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therapeutic interventions used to alleviate clinical disease.

Background

The zebrafish involved in this epizootic were wild-type and mutant strains used for the study of early embryonic development. Fry within the main facility were raised to breeding age to provide fertilized eggs. Progeny fish of various genetic backgrounds were maintained in this facility. The water system housing the zebrafish consisted of a commercially available recirculating system with mechanical (particulate), chemical (charcoal), UV, and biological filtration. The system used a reverse osmosis (RO) water supply with mineral salt supplementation (Instant Ocean by Aquarium Systems, Inc., Mentoe, Ohio) and contained approximately 35,000 zebrafish in 2,800 individual tanks ranging in size from 1 to 4 L. Approximately 10% of the water volume was replaced daily. The water system had been operational for more than three years, and the biological filter was well established. This was confirmed by daily monitoring of ammonia and nitrite concentrations that were consistently within acceptable limits. Maximal stocking density was about 30 adult fish/2 L of water, although most fish were maintained at a lower density. Light cycle consisted of a 14/10-h light/dark cycle. The fish were fed powdered flake feed (Tetramin by Tetra, Blacksburg, Va.), freeze-dried krill, and freshly hatched brine shrimp twice daily.

A separate water system in another location was used to quarantine fish arriving from outside sources. Fertilized eggs were collected from quarantined fish, treated with a bleach solution, and maintained in a quarantine incubator. At five days of age, fry were transferred to the main facility. These established genetic lines in the main facility were then used for various crosses to produce the fry. Fish from outside sources were not directly introduced into the main facility.

Clinical Outbreak

The epizootic in this facility began when sudden, high mortality in approximately 30-day-old fry was observed. One or more individual aquaria were found with 100% mortality on a near-daily basis. Often, mass mortality would occur overnight or between the early afternoon feeding and the late afternoon health check. Severely autolyzed carcasses with portions of the body missing were observed, and a substantial percentage of the tank population often was missing (no carcasses remaining). The acute presentation and high mortality of all fry implied a toxic event such as ammonia/nitrite/chlorine toxicosis or iatrogenic contamination with bleach or disinfectant soap due to improper sanitation of equipment. Renewed emphasis on sanitation procedures with regard to disinfection and cleansing of equipment was, therefore, instituted. However, this effort did not affect continued mortality of the fry.

Materials and Methods

Environmental analysis. Water quality parameters were monitored routinely within the facility. Basic water quality parameters were measured according to standard protocols and techniques (6). Normal water chemical analyses were performed routinely from different points within the facility. These analyses included ammonia (undetectable), nitrite (undetectable), nitrate (< 40 mg/dl), pH (6.8 to 8.0), temperature (28 ± 1°C), and conductivity (350 to 650 microsiemens); all were within normal limits for housing laboratory zebrafish. Wet mount preparations of various water samples from different points within the facility, including reservoir tank, biofilter bed, individual zebrafish tanks, and inflow piping, also were examined.

Figure 1. Photograph of representative zebrafish housing units used at this research laboratory and available through a commercial vendor. Fry were reared in individual 1- to 4-L capacity units. Water exchange occurs through afferent fresh water (black arrow) from above, with effluent draining through a mesh screen-covered orifice in the front of the unit (white arrow).

Necropsy. Euthanasia of zebrafish was accomplished by administration of an overdose of MS-222 (tricaine methanesulfonate, Finquel by Argent Chemical Laboratories, Redmond, Wash.). Fish were removed from the water five minutes after cessation of opercular movement. Fry were immediately examined under a dissecting microscope. Due to the small size of the fry, approximately 5 to 10 mm long, only limited gross examination of whole wet mount and impression smear preparations was conducted.

Histologic examination. Zebrafish fry were preserved in neutral-buffered 10% formalin. Longitudinal 5-µm-thick tissue sections of whole fry were examined, using hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Perl's iron, and Gram staining.

Water systems for fry culture. Several months prior to the epizootic, a new method of raising fry had been implemented in an attempt to reduce environmentally induced stress as a result of a high water flow rate. In addition to the physical effects of being trapped up against the outflow screen of tanks, fry expended large amounts of energy swimming against the water flow (Fig. 1). Although different methods of regulating a continual drip or low flow rate had been attempted previously, these often had inconsistent results since the water supply to a

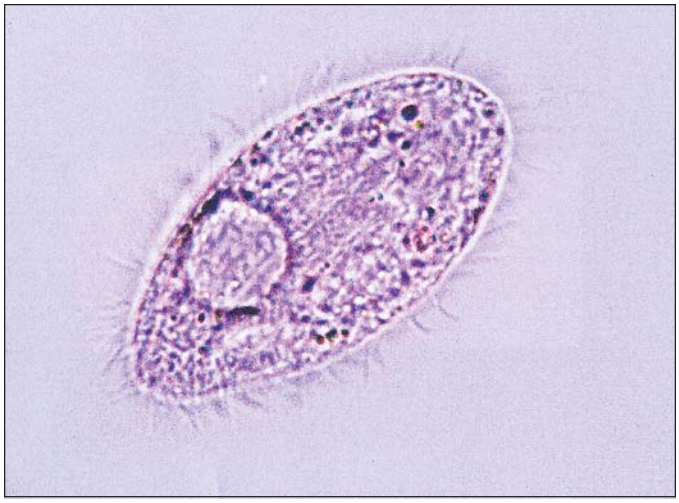

Figure 2. High-power photomicrograph from a wet mount preparation of the protozoan found in high numbers in water samples, as well as moribund and dead fry. This microorganism was an extremely motile, oval-shaped ciliate, approximately 30 µm in diameter, which moved with a characteristic 'spiraling football' motion. This ciliate had characteristics of morphology and behavior similar to those of *Tetrahymena* spp. Magnification, 1,000×.

particular tank could suddenly change when the supply to another part of the recirculating water system was adjusted. This would sometimes have drastic effects on young fry maintained on a continual drip or low-flow rate. In a further attempt to alleviate these 'environmental stressors,' the incoming water was turned off during most of the day, then on for a brief period twice daily. This new, 'off-water' method initially resulted in significantly higher survival rates of developing fry prior to the onset of the *Tetrahymena* sp.-associated epizootic.

Results

Environmental analysis. Repeated analysis of numerous water samples during the epizootic revealed basic water quality parameters within normal tolerances except for high nitrate values of 40 to 70 mg/dl that were observed on repeated measurements in fry-holding tanks. Examination of wet mount preparations of samples obtained from the reservoir and inflow piping was unremarkable. However, examination of samples from the biofilter and individual fry-holding tanks revealed high amounts of organic debris when evaluated by use of wet mount microscopy. Also, many wet mount samples contained more than three protozoan species as well as several fungal species identified sporadically. The most common protozoan found in high numbers in the water samples and associated with the moribund, and dead fry was an extremely motile, oval-shaped ciliate (Fig. 2). This protozoan was approximately 30 µm in diameter and moved with a characteristic 'spiraling football' motion. The morphology and behavioral characteristics were consistent with those of *Tetrahymena* spp.

Necropsy. Dissection of individual specimens was not conducted due to the small size of the affected fry. On examination of moribund or dead fish under a dissecting microscope, visible autolytic changes were seen on arrival at the diagnostic laboratory. One fry tank in particular being maintained in static conditions during most of the day was visually inspected during routine feeding and contained clinically healthy fry. Two hours later, 100% mortality of fry in the tank was detected. Remains of three fry were submitted for diagnostic evaluation. Immediate examination of several carcasses under the dissecting microscope revealed an abundance of protozoa clustered around food debris and fry carcasses. In three carcasses, the ventral body wall and eyes were absent. Two of the fry had no remaining viscera, and the gut of the third was being effaced by a swarm of protozoa. The protozoa were observed swarming inside the coelom of the carcasses and appeared to be feeding on the fry from within the coelom.

Histologic examination. Individual fry retained thin body outlines filled with fractured autolytic internal structures. Inside the body wall, numerous 12- to 30-µm-diameter oval parasitic organisms were distributed in deep and superficial locations (Fig. 3a). The organisms located in the deeper tissues were 12 to 15 µm in diameter, basophilic in response to H&E staining, uniformly PAS positive, and contained more than 10 to 15 tightly packed oval subunits, each having a barely discernible single densely basophilic central focus (Fig. 3a). Larger 20- to 30-µm organisms were regionalized to the superficial tissues and often formed distinct subepithelial nests of four to 10 organisms. These organisms contained eight to 10 larger, less tightly packed, discrete oval subunits with a variegated staining pattern, including brightly eosinophilic, densely basophilic, and black subunits (Fig. 3a). Only the basophilic subunits were PAS positive, and hemoglobin was not detected, using Perl's iron stain. Individual organisms with a holotrichal pattern of fine cilia were occasionally found on the epithelial surface above focal subepithelial clusters, suggesting recent emergence from the subepithelial location (Fig. 3b). Some subepithelial nests that were within a densely basophilic debris zone consistent with degenerating body wall musculature had a domed contour (Fig. 3c). The general morphology of the organisms was consistent with that of *Tetrahymena* sp. Gram staining revealed dense, mixed, gram-negative bacterial colonization along the outer surface of the body wall and throughout the body coelom consistent with terminal or postmortem overgrowth.

Control of the outbreak by modification of environmental conditions for fry culture. Initial measures were undertaken to improve the water quality in the fry tanks. For fry < 10 days of age, the number of water changes for the holding tanks was increased to four per day and the duration of each water change was increased from 10 to 20 min. Between days 10 and 15, fry were transitioned to a steady low-volume flow rate and, after day 15, fry were placed in a system with continuous flow of water. These changes were instituted to make the system less favorable for growth of the *Tetrahymena* sp. population. Given that higher salinity values are generally less facilitative to freshwater protozoan species growth, the salinity of the water was gradually increased to conductivity of approximately 1,000 microseimens.

Prior to the outbreak, fertilized eggs obtained from zebrafish matings in the main water system were not usually bleached prior to incubation. Although the water used for egg incubation was constituted from freshly distilled water and sea salts, protozoa were visible in the incubation dishes when viewed under a dissecting microscope at low magnification. It was, therefore, decided to bleach all eggs prior to incubation. Although bleach treatment did not eliminate the microorganisms, it substantially lowered protozoan numbers during the incubation period.

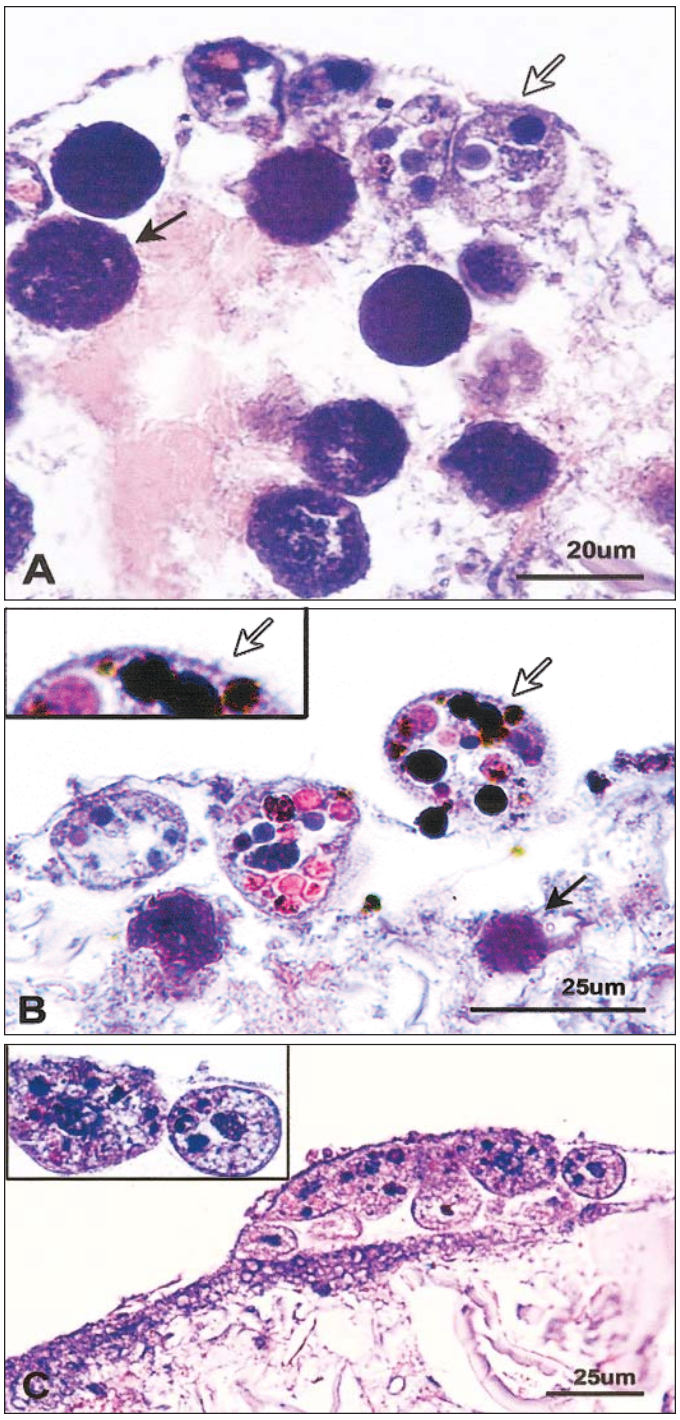

Figure 3. Infection of zebrafish tissues by a ciliated protozoan species: (A) Photomicrograph indicating a distribution pattern that includes basophilic organisms in deep tissues (closed arrow), and formation of subepithelial clusters of variegated forms (open arrow), H&E stain; magnification, 1,000×. (B) Photomicrograph of a section of the holotrichal pattern of fine cilia along the surface of a superficially located, 25-µm variegated organism (open arrow and inset). This emerging ciliate is subtended by a cluster of subepithelial variegated forms and deeper 12-µm, uniformly basophilic forms (closed arrow), H&E stain; magnification, 1,000×. (C) Photomicrograph of a subepithelial nest of variegated organisms (detail in inset) that creates a dermal pocket within the dense basophilic subepithelial layer. This layer appears to be degenerating body wall musculature, H&E stain; magnification, 400×.

Long-term improvements to the water system included increasing the wattage of the UV system from 45,000 to 200,000 microwatts/sec/cm^2 at a water flow rate of 20 gallons/min. Also, a program including increased sampling of culled fish to monitor for subclinical and clinical disease was implemented. Collectively, these changes resulted in termination of the epizootic plaguing the zebrafish fry culture.

Discussion

We hypothesize that *Tetrahymena* sp. was present as a part of the resident microflora within the biofilter of these recirculating water systems prior to the epizootic. With the changes implemented in husbandry practices concerning the rearing of young fry, an unexpected increase in organic loading due to fish excretions, excess food, and high nitrate concentration contributed to a shift in the character of the microbial flora within the water system. With a readily available food source and a favorable micro-environment in the fry tanks, the usually non-pathogenic *Tetrahymena* sp. in the water system in low numbers rapidly multiplied and resulted in the epizootic.

Treatment of fish superficially infected with *Tetrahymena* spp. can be attempted with the usual array of protozoan parasiticides (e.g. formalin and increased salinity), but is frequently unrewarding due to the debilitated state of the host (14). Topical treatment with immersions and baths is ineffective in treating systemic infections. Similar to our findings, others have reported that control of *Tetrahymena* spp. and other protozoa is best achieved by maintaining optimal water quality parameters, maintaining adequate nutrition, and removing excess organic debris, such as uneaten food (6, 14, 15).

Approximately twenty-four months after changes in management and husbandry practices, such as increased salinity and number of water changes, were implemented, appreciable fry mortality that characterized the original epizootic have not been observed. Although there was no indication of system-wide water quality issues involving adult zebrafish or other water systems, it was believed that the changes instituted for fry culture would also improve environmental conditions for the entire zebrafish population. However, protozoa with *Tetrahymena*-like morphology were still frequently observed in wet mount preparations and continue to be established microfauna in the water system.

In conclusion, results of this study highlight the need to monitor and maintain water quality on a routine basis and emphasize the fact that fry of egg-laying (oviparous) fish species like zebrafish are indeed susceptible to systemic *Tetrahymena* spp. infection that can result in high mortality.

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