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Protective immunization against *Tetrahymena* sp. infection in guppies (*Poecilia reticulata*)

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ABSTRACT

Systemic tetrahymenosis constitutes a serious problem in guppy (*Poecilia reticulata*) production worldwide and no therapeutic solution is available for this disease. Three immunization trials were conducted, testing the effectiveness of different *Tetrahymena* preparations applied by intraperitoneal injection (IP) with or without Freund's complete adjuvant (FCA) and with or without booster dose. In trial 1, immunization with the pathogenic Tet-NI 6 lysate and live attenuated Tet-NI 1 did not provide significant protection from infection, although infection rates were significantly lower in the Tet-NI 6-immunized group than in controls. In trial 2, mortality in Tet-NI 6 + FCA-immunized fish was 10%, significantly lower than in all other treatment groups, including Tet-NI 6 lysate, live attenuated Tet-NI 1 and controls (77, 67 and 73%, respectively). In trial 3, the lowest mortality rates were obtained in the Tet-NI 6 + FCA + booster-immunized group (15%). These levels were lower but not significantly different from the non-boostered Tet-NI 6-immunized group (28%) and the groups immunized with Tet-NI 1, with and without booster (32 and 34%, respectively). Mortality in these four groups was significantly lower than in controls, including adjuvant- and PBS-injected groups (72 and 81%, respectively). Body homogenates of immunized fish immobilized *Tetrahymena* in-vitro, as compared to no or very little immobilization in controls. Lysozyme levels in the Tet-NI 6 + FCA + booster group were significantly higher than in all other treatments in trial 2 and controls in trial 3. There was no significant difference in anti-protease activity among the differently immunized fish. We conclude that immunization with *Tetrahymena* lysates in FCA confers a high degree of protection from infection, suggesting this preparation as a basis for vaccine development.

1. Introduction

Infection with *Tetrahymena* sp., an opportunistic histophagous ciliate, is an unresolved disease problem in guppy (*Poecilia reticulata*), a popular tropical aquarium fish. The genus *Tetrahymena*, comprising ciliated protozoa (Ciliophora) of the order Hymenostomatida, has been reported from a range of fish species, including the Atlantic salmon *Salmo salar*, the zebrafish *Danio rerio*, pristella (*Pristella maxillaries*), neontetra (*Paracheirodon innesi*), cherry barb (*Puntius titteya* Diraniyagala), angelfish (*Pterophyllum scalare*) and platyfish (*Xiphophorus maculatus*) [1–4]. However, infections are most commonly reported from guppies, hence the names Tet disease and "guppy killer parasite" [4,5].

Tetrahymena includes free-living, generally saprozoic ciliates that feed on particulate food, including bacteria [6]. Many species are facultative parasites while others may be obligate [7,8]. The most commonly reported infections are caused by *Tetrahymena corlissi* [6,9–11], which is able to invade the visceral cavity, leading to systemic infection. *Tetrahymena* is believed to infect wounded and/or weakened fish [1,12,13]. It has been suggested that the ciliate physically destroys the host tissues, and reaches the internal organs via the musculature [11,12]. Invasive *Tetrahymena* appears to be closely associated with the epidermis, moving very slowly, presumably physically destroying the tissue with the aid of its cilia [14]. Less invasive isolates appear to only superficially infect the skin and move much faster, as observed in skin wet mounts. Most reports describe a lack of inflammatory response and no evident host-associated response to the infection [2,6,11,14].

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Tetrahymena species that have been reported to infect fish include *T. corlissi* [1,12,13,15,16] and *T. pyriformis* [2]. Pimenta-Leibowitz et al. [4] reported that poor environmental and adverse physiological conditions predispose the fish to infections with *Tetrahymena* sp.

Currently, no therapeutic solution is available for this disease. Conventional treatments against protozoan infections are effective against superficial, but not systemic (*Tetrahymena*) infections. Salogni et al. [17] reported tetrahymenosis in guppies imported to Italy from Malaysia. Treatment with antibiotics and a disinfectant could not prevent mortality, which reached 60% [17]. Treatment with combined application of a Chinese herb mix and salt bath has been reported to be effective [18].

A non-pathogenic *Tetrahymena* sp. has been successfully used in immunizations against *Ichthyophthirius multifiliis* infection [19] but to the best of our knowledge, immunization against *Tetrahymena* infection has not been attempted. In the present work, we report on immunization against *Tetrahymena* infection in guppies by intraperitoneal (IP) injection of attenuated and invasive *Tetrahymena*, with and without adjuvant. Changes in humoral immune response were evaluated.

2. Materials and methods

2.1. Fish

Naïve guppies (0.4–0.6 g) were obtained from a commercial fish farm and maintained in a 100-L container equipped with aeration and submerged biological filters. Experiments were conducted in 30- and 10-L aquaria and 1-L beakers in three to six replicates. Aquaria were similarly equipped with aeration and submerged biological filters but only aeration was placed in the 1-L beakers. Fish were fed daily at 2% of their body weight (Tropical Orange, Tzemah, Israel). To maintain adequate water quality, submerged biological filters were used and water exchange of 40% was applied every other day. Water-quality parameters were monitored weekly. Ammonia, nitrite and nitrate were measured by visocolor kits (Macherey-Nagel, Germany); levels were maintained below 0.5 ppm for ammonia and nitrite, and nitrate levels were 5–10 ppm. The pH level was 7.6, as measured by a pH-meter (Eutech Instruments, Singapore). Dissolved oxygen was maintained at above 80% saturation, as determined by YSI 52-dissolved oxygen meter (YSI Incorporated, USA).

Fish were treated in compliance with the principles for biomedical research involving animals. The experimental protocol was approved by the Ben-Gurion University Committee for the Ethical Care and Use of Animals in Experiments, authorization nos. IL-67-11-2002 and IL-51-8-2008.

2.2. Tetrahymena maintenance

Fish infected with *Tetrahymena* sp. (Tet-NI), which were originally imported from Singapore in 2005 and diagnosed with *Tetrahymena* sp. during the quarantine stage at a commercial fish farm, were brought to the lab and stocked in 10-L aquaria. Comparative DNA barcode analysis was conducted and Tet-NI was suggested to be a new species of *Tetrahymena* [20].

In-vivo and in-vitro maintenance was conducted as described by Pimenta Leibowitz and Zilberg [14]. Briefly, in-vivo infection was maintained in two separate containers of about 8 and 100 L by regularly adding naïve fish to replace mortalities. For in-vitro culture, Tet-NI was aseptically isolated from the internal organs (excluding the gastrointestinal tract), skin lesions, gills or tail of infected guppies and transferred to RM-9 culture medium (consisting of protease peptone, tryptone, glucose, liver extract and di-potassium hydrogen phosphate) in a petri dish and incubated at 25 °C. Penicillin G (3 mg/L) and streptomycin sulphate (3 mg/L) were added to prevent bacterial growth. Sub-culturing was conducted weekly under sterile conditions without antibiotic in a sterile hood (ADS Laminar, France). For clone production, a single cell of *Tetrahymena* from culture was inoculated into fresh medium. *Tetrahymena* clones from less than 10 passages in-vitro were used for infecting fish in all experiments. As Tet-NI appears to lose pathogenicity under prolonged culture conditions (determined by IP infection studies in guppies), it is regularly passed through guppies. Two different isolates of *Tetrahymena* were used in this study and are referred to as Tet-NI 1, a culture-attenuated isolate, and Tet-NI 6, an invasive isolate, maintained in culture for less than 10 weeks (10 passages in culture). *Tetrahymena* attenuation was achieved by continuous in-vitro culture for over 12 months (about 50 passages in culture) and confirmed by comparative infection studies with attenuated and invasive isolates.

2.3. Tetrahymena preparation for immunization

Cultured Tet-NI, 3–4 days after passage to fresh RM-9 medium, was used for both immunization and challenge infection. The ciliate

Table 1
Experimental design and treatments applied.

Trial no.	No. of replicates	Fish/replicate (fish size in g)	Container	Treatments	Booster (weeks)	Challenge (weeks)[a]
1[b]	6	60 (aquaria) 8 (beaker) (0.47 ± 0.14)	30-L aquaria 1-L beakers	Tet-NI 6 lysates Tet-NI 1 live PBS (control)	2	2
2	6	30 (0.54 ± 0.19)	10-L aquaria	Tet–NI 6 lysates + adj Tet-NI6 lysates Tet-NI 1 live PBS (control)	4	6
3	4	30 (0.63 ± 0.31)	10-L aquaria	Tet-NI 6 lysates + adj Tet-NI 1 lysates + adj Adjuvant only Tet-NI 6 lysates + adj[c] Tet-NI 1 lysates + adj[c] PBS (control)	4 4 4 – – 4	4

[a] Time after booster or after initial immunization in non-boostered groups.
[b] Fish were immunized in 30-L aquaria and challenged in 1-L beakers.
[c] No booster injection was applied.