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Evaluation of guppy (*Poecilia reticulata* Peters) immunization against *Tetrahymena* sp. by enzyme-linked immunosorbent assay (ELISA)

Galit Sharon[a,1], Pulak R. Nath[b,1], Noah Isakov[b], Dina Zilberg[a,*]

[a] The Jacob Blaustein Institutes for Desert Research, Ben Gurion University of the Negev, Midreshet Ben Gurion, Israel
[b] The Shraga Segal Department of Microbiology, Immunology and Genetics, Ben Gurion University of the Negev, Beer Sheva, Israel

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ABSTRACT

Analysis of the effectiveness of guppy (*Poecilia reticulata* Peters) immunization based on measurements of antibody (Ab) titers suffers from a shortage of reagents that can detect guppy antibodies (Abs). To overcome this problem, we immunized mice with different preparations of guppy immunoglobulins (Igs) and used the mouse antisera to develop a quantitative enzyme-linked immunosorbent assay (ELISA). The most efficient immunogen for mouse immunization was guppy Igs adsorbed on protein A/G beads. Antisera from mice boosted with this immunoglobulin (Ig) preparation were highly specific and contained high Ab titers. They immunoreacted in a Western blot with Ig heavy and light chains from guppy serum, and Ig heavy chain from guppy whole-body homogenate. The mouse anti-guppy Ig was applied in an ELISA aimed at comparing the efficiency of different routes of guppy immunization against *Tetrahymena*: (i) anal intubation with sonicated *Tetrahymena* (40,000 *Tetrahymena*/fish in a total volume of 10 μL) mixed with domperidon, deoxycholic acid and free amino acids (valine, leucine, isoleucine, phenylalanine and tryptophan), or (ii) intraperitoneal (i.p.) injection of sonicated *Tetrahymena* in complete Freund's adjuvant (15,000 *Tetrahymena*/fish in total a volume of 20 μL). Negative control fish were anally intubated with the intubation mixture without *Tetrahymena*, or untreated. ELISA measurement of anti-*Tetrahymena* Ab titer revealed a significantly higher level of Abs in i.p.-immunized guppies, compared to the anally intubated and control fish. In addition, the efficiency of immunization was tested by monitoring guppy mortality following (i) i.p. challenge with *Tetrahymena* (900 *Tetrahymena*/fish) or (ii) cold stress followed by immersion in water containing 10,000 *Tetrahymena*/mL. Fish mortality on day 14 post-*Tetrahymena* infection by i.p. injection exceeded 50% in the control and anally intubated fish, compared to 31% in i.p.-immunized fish. Immunization did not protect from pathogen challenge by immersion. The results suggest a direct correlation between the anti-*Tetrahymena* Ab response and fish resistance to i.p.-injected *Tetrahymena*, but not to infection by immersion preceded by cold stress.

1. Introduction

Guppies (*Poecilia reticulata* Peters) are the most popular fish among hobbyists due to their vibrant colors and the fact that they are easy to breed and maintain (Harpaz

* Corresponding author. Tel.: +44 972 8 6596818; fax: +972 8 6596742.
E-mail address: dzilberg@bgu.ac.il (D. Zilberg).
[1] These authors contributed equally to this work and share first authorship.

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et al., 2005). Although they are successfully produced in intensive systems, this practice increases the occurrence of disease outbreaks. One of the most serious diseases affecting guppies is caused by *Tetrahymena* sp., a ciliated protozoan that is also known as the guppy killer parasite. There are many different species of *Tetrahymena*, with over 40 documented (Simon et al., 2008). They are facultative parasites that infect a large range of fish species, and they appear to be highly pathogenic, particularly in guppies (Imai et al., 2000; Ponpornpisit et al., 2000; Kim et al., 2002; Shenberg, 2003; Sharon et al., 2014a). In this study, we used a *Tetrahymena* strain reported by Pimenta Leibowitz and Zilberg (2009), which was identified as a new strain by Chantangsi et al. (2007), based on the sequence of its mitochondrial cytochrome c oxidase subunit 1 (cox 1), a gene proposed as a DNA barcode for the identification of animal species.

Initial immunization against this strain of *Tetrahymena* was reported by Chettri et al. (2009), demonstrating protection from experimental infection following intraperitoneal (i.p.) immunization with adjuvant. The in vivo protection from infection correlated with in vitro immobilization of the parasite by body homogenates of immunized fish, suggesting that the immunization induced the production of immobilizing antibodies (Abs) (Chettri et al., 2009). Iglesias et al. (2003) reported that immunization against another invasive parasitic ciliate, *Philasterides dicentrachi*, causing systemic scuticociliatosis in turbot [*Scophthalmus maximus* (Linnaeus)], induces antibody (Ab) production which correlates with protection from infection. In that work, crude extract and ciliary Ag fractions of the parasite were used as primary Ags, and Ab response was evaluated by ELISA. Elevated OD readings of the tested antiserum in the enzyme-linked immunosorbent assay (ELISA) correlated with the antiserum's ability to mediate parasite agglutination in vitro, demonstrating the effectiveness of the immunization and the expression of a surface-immobilizing Ag(s) on the parasite (Iglesias et al., 2003).

In the present study, we used two different routes to immunize guppies against *Tetrahymena*: anal intubatation and i.p. injection, and developed an ELISA to compare the efficiency of the consequent Ab response. Anal intubation was selected since the hindgut segment of teleosts has been found to be the main site of Ag uptake (Georgopoulou and Vernier, 1986). Introduction of Ag at this site was aimed at obtaining a proof of concept for the feasibility of immunization via the gastrointestinal tract.

IgM, the major Ab subclass in fish, is involved in the immune response against pathogens (Wilson and Warr, 1992; Pilström and Bengtén, 1996). However, IgM differs between fish species, and due to a lack of commercial guppy immunoglobulin (Ig) Abs, we prepared these Abs in mice to use them in a quantitative ELISA. The presence of IgM has previously been demonstrated in guppies by Lim et al. (2009), who also developed guinea pig anti-guppy Ig antiserum for an ELISA. In the present study, we used several different preparations of guppy Igs as immunogens, and following mouse immunization, compared the efficiency and specificity of the resulting antisera.

2. Materials and methods

2.1. Fish

Three-month-old guppies were obtained from commercial ornamental fish farms in the Arava valley, Israel. Fish were pre-examined to ensure absence of *Tetrahymena* sp. before initiating the study. Naïve fish were kept in 100-L tanks supplied with biological filters and aeration, and fed daily with commercial guppy food (Ocean Nutrition, Newark, CA) at about 2% of their body weight until the end of the experiments. Water quality was monitored weekly; ammonia and nitrite levels were measured by Visocolor® kits (Macherey-Nagel, Düren, Germany) and maintained at <0.5 ppm; water oxygen was measured using the YSI 52-dissolved oxygen meter (YSI Incorporated, Yellow Springs, OH) and maintained at >80% saturation. All procedures were conducted on anesthetized fish (250 μL clove oil/L of water) for >3 min). Experimental protocols were carried out in compliance with the principles of biomedical research involving animals, set up by the Ben Gurion University Committee for the Ethical Care and Use of Animals, authorization number: IL-51-8-2008.

2.2. Isolation and maintenance of Tetrahymena

The *Tetrahymena* sp. used in the experiments was originally diagnosed in guppies imported from Singapore at the quarantine stage. Comparative DNA barcode analysis of the cox 1 gene carried out by Chantangsi et al. (2007), indicated that the isolated *Tetrahymena* is a new species. *Tetrahymena* was maintained in vivo and in vitro as described by Pimenta Leibowitz and Zilberg (2009). Briefly, infected fish were maintained in 100-L containers, and naïve fish were added regularly to replace mortalities. For in vitro culture, *Tetrahymena* sp. isolated from infected fish organs (skin, gills or tail) was placed in RM9 medium [containing 5 g protease peptone, 5 g tryptone, 2 g glucose, 0.1 g liver extract and 0.2 g dipotassium hydrogen phosphate in 1 L of double-distilled water (DDW); ATCC, 1999]. Subculturing was conducted once a week and a passage through fish was performed once every 8–10 in vitro passages, by i.p. injection of ca. 900 Tetrahymena in 20 μL RM9 medium per fish, to maintain the parasite's pathogenicity, followed by reisolation and culture.

2.3. Demonstrating Igs in guppy serum and body homogenate

2.3.1. Blood withdrawal and serum preparation

Blood was withdrawn from large (1.5–2.0 g) female guppies, immunized (70 fish) or not (70 fish) with glutathione *S*-transferase (GST; GE Healthcare Bio-Sciences, Uppsala, Sweden), a well-established antigen, which is known to induce a high Ab response in fish (Oshima et al., 1996). For immunization, female guppies were injected i.p. with 350 μg GST in PBS (pH 7.2, 0.05 M) emulsified in complete Freund's adjuvant (CFA; Sigma, St. Louis, MO), at a ratio of 1:2 (final volume of 20 μL/fish). After 3 weeks, fish were boosted with GST emulsified in incomplete Freund's adjuvant (IFA; Sigma) and blood was withdrawn 4 weeks