Prophylactic Effects of Chemicals and Immunostimulants in Experimental Tetrahymena Infections of Guppy

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ABSTRACT—The prophylactic effects of chemicals and immunostimulants on the Tetrahymena infection of guppies (Poecilia reticulata) were studied through experimental infections. Tetrahymena pyriformis was used in an experimental infection on a guppy population pre-treated by the following method; the base of the caudal fin was covered with a 10% acetic acid-soaked cotton strip for 3 min. The study consisted of screening anti-Tetrahymena chemicals, testing prophylactic effects of selected chemicals or immunostimulants, and finally evaluating the combination effects of the chemicals and immunostimulants. The single uses of chemicals or immunostimulants did not completely prevent the Tetrahymena infection. Prevention was attained by the combination of a 0.5% sodium chloride bath and a feeding of C-UPIII (a Chinese herb mix) diet.

Key words: Tetrahymena pyriformis, guppy, Poecilia reticulata, ciliate, immunostimulant, prophylaxis, experimental infection

Guppies Poecilia reticulata are perhaps the most popular ornamental fish, recommended as perfect species for beginners with an interest in aquatic hobbies. Over the past few years, however, the Tetrahymena infection, or so-called Tet disease, has become widespread in home aquaria, pet shops and commercial farming ponds. Tetrahymena belongs to the phylum Ciliata, of which all the species are involved in facultative parasitism to some extent (Corliss, 1960). In the fish infected with Tetrahymena, external signs are lethargic swimming, eroded skin and fins, and a swollen gill cover, all reducing the overall commercial value of the fish. Prolonged infection is eventually fatal (Imai et al., 2000). Although many chemical substances have been introduced against various parasitic diseases as control measures (Tojo and Santamarina, 1998; Madsen et al., 2000), there has been no successful control of the Tetrahymena infection.

In our previous study (Ponpornpisit et al., 2000), we have reported on the experimental infection of Tetrahymena pyriformis on guppy populations. The report revealed that the experimental infection did not succeed in very low Tetrahymena cell densities in ambient water and in the fish with mild skin damage, suggesting that chemoprophylaxis and immunoprophylaxis are prospective control measures against Tetrahymena infections. This study is aimed at examining the prophylactic effects of chemicals and immunostimulants in experimental infections with Tetrahymena in order to establish the successful control and prevention.

Materials and Methods

Fish

Guppies (2.5–3.0 cm in body length) were used in all experiments. They were purchased from a pet shop in Miyazaki, southern Japan. For the experimental infections, a formalin bathing at 25 ppm for 24 h was employed on the fish to eradicate pathogenic agents. They were then kept in a 150 L tank and supplied with commercial feed twice a day for one week prior to the experiments. In all experiments, 10 fish were allocated for each test.

Tetrahymena culture

The ciliata Tetrahymena used in this study were iso-
lated from lesions of a naturally infected guppy. They were identified as *T. pyriformis* according to our previous report (Ponpornpisit et al., 2000). They were cultured and maintained in a *Tetrahymena* culture medium (ATCC culture medium 357, 10801 University Boulevard, Manassas, VA) at 25°C and subcultured every 3 weeks by adding 1 mL of the old culture medium to 10 mL of fresh medium. One day before the experiments started, 10 mL of the *Tetrahymena* culture medium was transferred to 500 mL of fresh medium. Immediately before the start of the experiments, the densities of stationary phase cells were adjusted in accordance with filtered water.

**Experimental infection with Tetrahymena on guppies**

The experimental infection was done according to the method described in our previous report (Ponpornpisit et al., 2000). This method promises a 80 to 100% infection rate in all the trials. In brief, fish were anesthetized, wounded at the base of the caudal fin by covering it with a 10% acetic acid-soaked cotton strip for 3 min (Ponpornpisit et al., 2000), and then exposed to 800 cells/mL of Tetrahymena suspension for 24 h. The exposure was carried out, in aerated 4 L of water using a 10 fish/tank at 25°C and pH 7.0. The numbers of infected fish were recorded at 24 h after exposure to the Tetrahymena suspension. Infection was then confirmed under a microscope by the appearance of Tetrahymena cells underneath the skin of the caudal fin.

**Experimental diets**

As for the experimental diets used in this study, their components are shown in Table 1.

**Experimental design and conditions**

This study consisted of four experiments. The first experiment involved the screening of anti-Tetrahymena chemicals. In the second and third experiments, the prophylactic effects of the selected chemicals and immunostimulants were separately examined in the guppy populations experimentally infected with *Tetrahymena*. The fourth experiment attempted to combine the selected chemicals and immunostimulants for the successful control of the experimental Tetrahymena infection. In all the experiments, the water temperatures, dissolved oxygen and pH levels were maintained at 25°C, > 5 ppm and 6.8–7.2, respectively.

**Experiment I. Screening of anti-Tetrahymena chemicals**

Twelve tested chemicals were chosen from the viewpoint of anti-parasitic action from several reports (Schaperclaus et al., 1992; Lipton, 1993; Novotny et al., 1996). They were categorized into dyes (acriflavine, methylene blue and malachite green), antibiotics (metronidazole, chloramphenicol and oxytetracycline), helminthicides (praziquantel), protothecicides (quinine sulfate), disinfectants (ammonium hydroxide, formalin and hydrogen peroxide) and salts (sodium chloride). One hundred mL water containing free-living Tetrahymena cells was prepared in flasks with densities of 640 cells/mL in Trial 1 and 880 cells/mL in Trial 2. Each of the chemicals was added to the Tetrahymena suspension in three concentrations. Two mL of the test water from each flask was sampled at 0, 1, 3, 24 h, and the numbers of living Tetrahymena cells were counted.

**Experiment II. Effects of selected chemicals in experimental infections with Tetrahymena**

Sodium chloride and hydrogen peroxide were selected based on the results from Experiment I as mentioned later. Fish were exposed to Tetrahymena suspensions supplemented with various concentrations of chemicals as shown in Fig. 3. The control test was prepared without any chemical additives. After the experimental infection, the infection rate was recorded in each of the tests and two living fish or newly dead fish were taken as samples from each test in order to investigate the side effects of the chemicals. They were fixed in Bouin's fluid and subjected to light microscopic observations with a routine paraffin section technique using hematoxylin-eosin stain. When the experiment finished, feeds were supplied to the test fish in order to see their appetite. This experiment was duplicated.

**Experiment III. Prophylactic effects of immunostimulants in experimental infections with Tetrahymena**

The prophylactic effects of two immunostimulants,

### Table 1. Composition of the experimental diets (%)

<table>
<thead>
<tr>
<th>Compositions</th>
<th>Control diet</th>
<th>Glucan diet</th>
<th>C-UPIII diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown fish meal</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>α-Starch</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Dextrin</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cellulose</td>
<td>2</td>
<td>1.8</td>
<td>1.5</td>
</tr>
<tr>
<td>C-UPIII (Chinese herb mix, Yamanouchi Co. Ltd.)</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucan (Yeast origin, Wako Co. Ltd.)</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Mineral mixture (MacCollum’s no.185 protocol)</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Vitamin mixture (Halver's protocol)</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
glucan and C-UPIII against the *Tetrahymena* infection were examined in this experiment. A glucan or C-UPIII diet was administered to fish *ad libitum* twice a day. After 10 days of feeding, the fish were subjected to the experimental *Tetrahymena* infection. Data record and histopathological analysis were similar to Experiment II. This experiment was duplicated.

**Experiment IV. Combination of chemoprophylaxis and immunoprophylaxis for the successful control against experimental *Tetrahymena* infections**

In this experiment, after the fish were fed the C-UPIII or control diets *ad libitum* twice a day for 10 days, the experimental infection was administered. Fish were exposed to *Tetrahymena* in water containing 0.5% sodium chloride or 200 ppm hydrogen peroxide during the entire infection period. In the control tests, no chemicals were added to the *Tetrahymena* water. The infection rate in each test was recorded after experimental infection for 24 h. This experiment was duplicated.

**Statistical analysis**

In the experiments II, III, IV, data obtained from two tanks in each experimental group were summed up and statistically analyzed by Fisher’s exact possibility test, as any significant difference was not detected between the tanks in each experimental group (Fisher’s exact probability test, P<0.05).

**Results**

**Experiment I. Screening of anti-*Tetrahymena* chemicals**

The chemicals listed in Fig. 1 little decreased the cell density of *Tetrahymena*. However, malachite green, sodium chloride, ammonium hydroxide, formalin, quinine sulfate and hydrogen peroxide apparently decreased the cell densities, showing clear anti-*Tetrahymena* effects. Since the results were similar in the 2 trials for each chemical tested, the results of trial 1 were shown in Fig. 2.

**Experiment II. Effects of selected chemicals in experimental infections with *Tetrahymena***

Sodium chloride and hydrogen peroxide showed prophylactic effects in experimental infections with *Tetrahymena* (Fig. 3). Although 400 ppm hydrogen peroxide solution reduced the infection rate to its lowest point, it killed all the fish before the test was terminated. A histopathological survey revealed that 400 ppm hydrogen peroxide solution caused serious hemolysis in blood cells and necrosis in the gills and skin (Fig. 6). Both 0.5% sodium chloride and 200 ppm hydrogen peroxide solution created slight hyperplastic changes in the gill and skin, but did not repress fish appetite under our observation.

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**Fig. 1.** Non-effective chemical agents against *Tetrahymena* free-living cells. In each the chemical, the data of the highest concentrations are shown. ◊, control; □, acriflavine 20 ppm; △, methylene blue 40 ppm; ×, metronidazole 40 ppm; *, chloramphenicol 400 ppm; ○, oxytetracycline 400 ppm; †, praziquantel 500 ppm.
Fig. 2. Effective chemical agents against Tetrahymena free-living cells. Symbols □, ■, △ and × represent control, low, middle and high concentrations of the chemicals tested. They are 0, 0.1, 0.2 and 0.3 ppm for malachite green, 0, 0.25, 0.5 and 1% for sodium chloride, 0, 50, 100 and 200 ppm for ammonium hydroxide, 0, 50, 100 and 200 ppm for formalin, 0, 100, 200 and 400 ppm for quinine sulfate, and 0, 100, 200, and 400 ppm for hydrogen peroxide.

Fig. 3. Infection rates of guppies with Tetrahymena in the chemoprophylaxis test. *; Significant difference from the control (P<0.05).

Experiment III. Prophylactic effects of immunostimulants in experimental infections with Tetrahymena

In the experimental infection using Tetrahymena, two immunostimulants, glucan and C-UPIII, drastically decreased the overall infection rate (Fig. 4). In the histological analysis, inflammatory responses were quite apparent in the wounded lesions of tested fish (Fig. 7). However, the inflammatory responses were scarce in the control fish (Fig. 8).
Experiment IV. Combination of chemoprophylaxis and immunoprophylaxis for the successful control against experimental Tetrahymena infections

Effects of C-UPIII feeding in a combination with 0.5% sodium chloride or 200 ppm hydrogen peroxide were examined experimentally. The combination of the 0.5% sodium chloride bath with the C-UPIII feeding attained complete control of experimental Tetrahymena infection (Fig. 5).

Discussion

In screening of anti-parasitic chemicals on Experiment I, six chemicals had the anti-Tetrahymena effects. However, only sodium chloride and hydrogen peroxide were selected as candidates for chemoprophylaxis tests of Experiment II, because harmful chemicals to humans and other organisms should be avoided in practical use. Both of the chemicals showed prominent prophylactic effects in the experimental infection with Tetrahymena. However, it appears that complete control using chemical agents is followed by serious side effects on the fish, judging from the high mortality and histological damages on the tested fish exposed to 400 ppm hydrogen peroxide solution. Immunoprophylaxis was also examined in Experiment III, according to the suggestion in our previous report (Ponpornpisit et al., 2000). Glucan and C-UPIII employed in this study have been known to improve the non-specific immune system in fish (Chansue et al., 2000). In the experimental infections with Tetrahymena, very low infection rates were induced by both the immunostimulants. Our previous study (Ponpornpisit et al., 2000) recorded that inflammatory responses were scarce in natural and experimental Tetrahymena infections. This was true in the instance of the control fish in this experiment, whereas the inflammatory responses stood out in the tested fish with the immunostimulant feeding. Thus, the drastic prophylactic effects appear to be established by immunostimulants through an improved immune system. Nevertheless, this immunological method did not bring complete prophylaxis.

The single use of anti-Tetrahymena chemicals and immunostimulants did not attain the successful control of experimental Tetrahymena infection in Experiments II and III. However, a combination of a chemical bath
with an immunostimulant feeding seemed to be worth trying in Experiment IV, allowing for a few side effects. Both 0.5% sodium chloride and 200 ppm hydrogen peroxide solution were chosen as the test chemicals, because Experiment II revealed their excellent prophylactic effects without serious side effects. C-UPIII has been known to be more effective as a stimulant than glucan (Chansue et al., 2000). Therefore, the combinations of 0.5% sodium chloride or 200 ppm hydrogen peroxide bath with C-UPIII feedings were thought to be a good combination. As a result, the combination of 0.5% sodium chloride bath with C-UPIII feedings was the best treatment for the complete control of experimental infection with *Tetrahymena*. In this combination treatment, further studies should be needed for practical use in naturally infected guppies with *Tetrahymena*.

Acknowledgment

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References


Fig. 6. Skin of fish exposed to 400 ppm hydrogen peroxide for 1 day after experimental infection in experiment II. Necrosis is observed in the skin (arrow). H&E stain.

Fig. 7. Skin of C-UPIII-fed fish after the experimental infection in experiment III. The epidermis was exfoliated and necrotized. Inflammatory cell infiltration (arrow) with edema is observed in the injury site. H&E stain.

Fig. 8. Skin of control fish exposed to a *Tetrahymena* suspension in the experimental infection. Necrosis without inflammatory cells infiltration is observed. H&E stain.