

Some Growth Characteristics of *Tetrahymena* sp., a Parasite of Guppy, *Poecilia reticulata* and the Effect of a Mixture of Two Commonly used Protozoocides on its Population Density

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Abstract

Various species and strains of *Tetrahymena* have a potential for being more virulent especially when the water is high in organic matter and fish are immunologically depressed causing "Tetrahymenosis". An outbreak of Tetrahymenosis was experienced by Sri Lankan guppy farmers in the recent past for which different treatments were employed indiscriminately. Present study investigated some growth characteristics of this fish parasite, which could be useful in import risk analysis and in screening female brooders of guppy. *Tetrahymena* sp. tested multiply rapidly in the presence of freshly killed guppy (as the substrate) and chloramphenicol $50 \mu\text{g ml}^{-1}$ with a generation time of 2.6 hours at 28°C . Combined effect of methylene blue (2 mg l^{-1}) and Zinc free malachite green (0.03 mg l^{-1}) completely inhibited *in situ* multiplication of *Tetrahymena*.

Introduction

Poecilia reticulata, commonly known as guppy is a popular tropical ornamental fish of which different varieties account for a greater proportion of the total freshwater ornamental fish export from Sri Lanka. In the recent past, guppy farms in Sri Lanka experienced an outbreak of a disease known as "guppy disease", "Tet disease", "Tetrahymenosis", or "guppy killer disease", which caused high mortalities resulting in significant economic losses (Hettiarachchi and Hettiarachchi 1999; Hettiarachchi and Amararatunga 2000). Southgate (1993) and Gratzek

(1993) described the causative agent of "Tet disease" as a *Tetrahymena* species, a normal free living, freshwater, polysaprobic, opportunistic, ciliate parasite. Gratzek (1993) and Butcher (1993) reported that various species and strains of *Tetrahymena* have a potential for being more virulent especially when the water is high in organic matter and fish are immunologically depressed. According to Lin et al. (1993) *T. pyriformis* is a free-living, non-pathogenic ciliated protozoan that occasionally becomes parasitic on live-bearing fish. *T. corlissi* is more pathogenic and guppies and other live bearers are the most susceptible species to this parasite. Infestation of *T. corlissi* produces signs of necrosis and hemorrhagic areas on the skin of fish and foci of parasites have been found in kidneys, brain and muscles (Butcher 1993). Laoprasert et al. (2002) also reported on "Tetrahymenosis" in guppy while Wakita et al. (2002) reported on Tetrahymenosis in dwarf gourami indicating that the parasite could be a threat to even egg layers.

During the outbreak, many Sri Lankan guppy farmers treated the infected guppies with different chemicals and broad-spectrum antibiotics such as chloramphenicol indiscriminately. However, farmers themselves have observed that severity of the disease increased with the use of antibiotics indicating that there exist an interaction between *Tetrahymena* and bacteria. Literature on growth characteristics of *Tetrahymena* sp. are scarce and therefore this work was undertaken to study some growth characteristics of this parasite and to develop methods to amplify population density (for quarantine purposes) and to control it which could be useful in the health management of guppy populations.

Materials and Methods

Stock culture of *Tetrahymena* sp.

Ten dead *Poecilia reticulata* which were heavily infected with *Tetrahymena* sp. were collected from a commercial guppy farm and stored in 200 ml of water at 4-6°C as the stock culture. This stock culture was vigorously shaken before taking inocula. Wet mounts prepared from skin lesions of infested guppy were observed under the microscope and the parasite was identified to generic level according to Jahn (1970).

Growth characteristics of *Tetrahymena* sp.

Effect of different culture conditions on population density

Freshly killed uninfected *Poecilia reticulata* measuring about 1.5 cm in total length (TL) were used as the substrate for *Tetrahymena* sp. Five different culture conditions (A, B, C and D) and a control (E) were

Growth characteristics of Tetrahymena

arranged in triplicate as shown in Table 1 and were inoculated with 100 μ l of stock culture of *Tetrahymena* sp.

Table 1. The different culture conditions provided for *Tetrahymena* sp. E is the control culture condition.

Treatment	Culture condition				
	A	B	C	D	E
Culture volume (ml) (aged tap water)	10	10	10	10	10
Freshly killed one guppy as substrate (1.5 cm in TL)	+	+	-	-	-
Freshly killed and autoclaved 121°C one guppy as substrate (1.5 cm in TL)	-	-	-	+	-
Freshly killed and macerated one guppy as substrate (1.5 cm in TL)	-	-	+	-	-
Incubating temperature °C	28± 1	4±1	28±1	28±1	28±1
<i>Tetrahymena</i> inoculum	+	+	+	+	+
+ provided					
- not provided					

After shaking the culture vigorously, eight samples of 50 – 100 μ l (for counting convenience) were taken from each vial with a variable micropipette (1-1000 μ l Pipetteman) at 24 hour intervals for 72 hours and the number of organisms were counted under the light microscope using one drop of 2% formalin to kill the organisms.

Effect of chloramphenicol on the population density of Tetrahymena sp.

Twelve *Tetrahymena* cultures were arranged providing the conditions that supported the maximum population density and four different concentrations of chloramphenicol each with 3 replicates (25, 50, 100 and 200 μ g/ml) were added to these cultures separately. This was done to find out the best antibiotic concentration that inhibits competing bacterial populations in the culture. The cultures were then inoculated with an equal number of *Tetrahymena* sp. Eight samples of 25 – 100 μ l from each culture (depending on the age of the culture) were taken at 24 hour intervals after shaking the culture vigorously and the *Tetrahymena* was enumerated as in the previous experiment. The generation time was calculated for the exponential phase of the culture which had the highest growth rate using t/n , where t is the time in hours and n is the number of generations during

the exponential growth phase which was obtained from the equation $n = \log N - \log N_0 / \log 2$ where N is final cell number, and N_0 is initial cell number (Madigan et al. 1997).

Growth curve of clones of Tetrahymena sp.

Three vials, each containing aged tap water with $50 \mu\text{g ml}^{-1}$ chloramphenicol (the concentration which gave the maximum population density of *Tetrahymena* sp.) and freshly killed *P. reticulata* were inoculated separately with one cell of *Tetrahymena* sp. using micromanupulation. Eight samples of culture medium ($25 - 100 \mu\text{l}$, depending on the age of the culture) were withdrawn from each vial at 24 hour intervals for 15 days and the *Tetrahymena* population was enumerated. A growth curve was plotted using the mean number of *Tetrahymena* sp. recorded at each sampling interval.

Effect of a mixture of two commonly used protozoacides on the population density of Tetrahymena sp.

Composition of the culture medium arranged to investigate the effect of two protozoacides commonly used in ornamental fish culture, namely methylene blue and zinc free malachite green oxalate is given in Table 2; the concentrations of the two protozoacides were chosen after series of preliminary tests. Here, three replicates were arranged with the protozoacides and the control (without protozoacides), which also had three replicates. Eight $100 \mu\text{l}$ samples were withdrawn from each replicate at 24 hour intervals for seven days and the presence or absence of *Tetrahemena* sp. in the samples were observed under the microscope.

Table 2. Composition of the culture medium arranged to investigate the effect of protozoacides on population density of *Tetrahymena* sp.

Component	Amount/Concentration
Aged tap water	10 ml
Chloramphenicol ($50 \mu\text{g ml}^{-1}$)	500 μg
Substrate	one dead guppy (1.5 cm TL)
Inoculum (stock culture)	100 μl
Zinc free malachite green oxalate (0.03 mg l^{-1})	0.0003 mg
Methylene blue (2.00 mg l^{-1})	0.02 mg

Results

Two rows of cilia, which began at the mouth (oral groove) and extended posteriorly, were observed on *Tetrahymena* sp. under the light microscope. A long single cilium was also observed among the cilia at the posterior end of the organism.

Infected guppies showed white patches just in front of the dorsal fin and haemorrhagic lesions on the caudal peduncle. Freshly killed whole guppy at ambient temperature ($28 \pm 1^\circ\text{C}$; Treatment A) gave significantly higher population density of *Tetrahymena* sp. than in other treatments ($P < 0.05$) throughout the 72 hour period while the lowest population density was recorded in the culture containing macerated substrate (Treatment C; Figure 1).

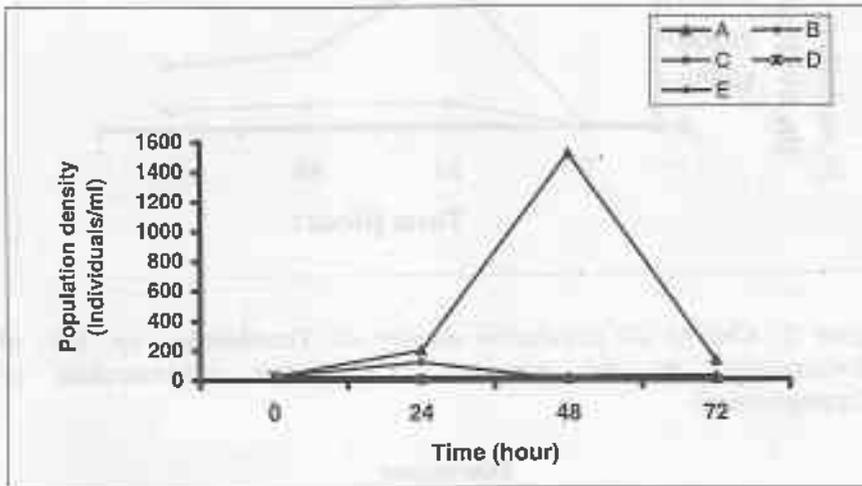


Figure 1. Change of the population density of *Tetrahymena* sp. with time under different culture conditions (for A, B, C, D, and E, refer Table 1).

Chloramphenicol at the concentration of $50 \mu\text{g ml}^{-1}$ supported the highest population density of *Tetrahymena* sp. ($P < 0.01$) when combined with the best culture conditions (freshly killed whole guppy as the substrate at ambient temperature). The maximum density (36,000 individuals/ml) was achieved 24 hours after inoculation and the generation time of the population during the exponential phase was 2.6 hours. Chloramphenicol at $200 \mu\text{g ml}^{-1}$ completely inhibited the growth of the population of *Tetrahymena* sp. while $25 \mu\text{g ml}^{-1}$ chloramphenicol had supported a considerable increase in the number of *Tetrahymena* sp. (Figure 2). Figure 3 shows the growth curve of clones of *Tetrahymena* sp. observed during the

15 day culture period. The maximum density of the parasite in clones was reached 11 days after inoculation.

Tetrahymena sp. was absent in all the samples taken from the cultures containing the mixture of chemicals, zinc free malachite green oxalate (0.03 mg l^{-1}) and methylene blue (2.0 mg l^{-1}), while it was present in the samples taken from the cultures without the chemicals.

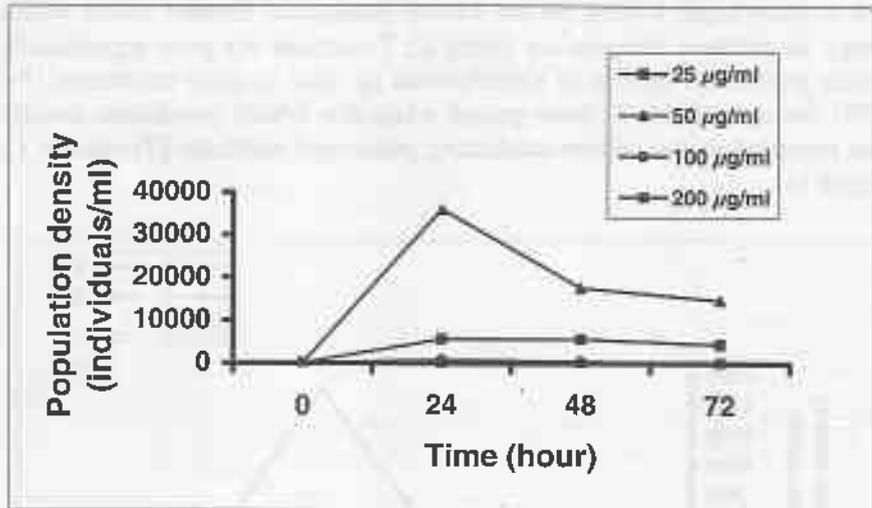


Figure 2. Change of population density of *Tetrahymena* sp. (no. of individuals/ml) in the presence of different concentration of chloramphenicol.

Discussion

Presence of two rows of cilia, which begin at the oral groove and extended posteriorly confirmed that the ciliate in the present study belongs to the genus *Tetrahymena* (Jahn 1970). Results of the present study suggest that freshly killed unprocessed tissues of *Poecilia reticulata* support a good growth of *Tetrahymena* sp. *in situ* at ambient temperature of $28 \pm 1^\circ\text{C}$. According to Lin et al. (1993), *T. pyriformis* could be cultured aerobically in an enriched medium. Significantly higher population density of *Tetrahymena* sp. ($P < 0.01$) that resulted in the culture medium containing $50 \mu\text{g ml}^{-1}$ of chloramphenicol suggests that the antibiotic has suppressed the competing bacterial populations in the medium allowing the ciliate to multiply rapidly. It has also been reported that the use of novobiocin (an antibiotic) had increased the number of individuals of *Tetrahymena* sp. causing increased mortality in infected guppies (Ponpornpisit et al. 1998).

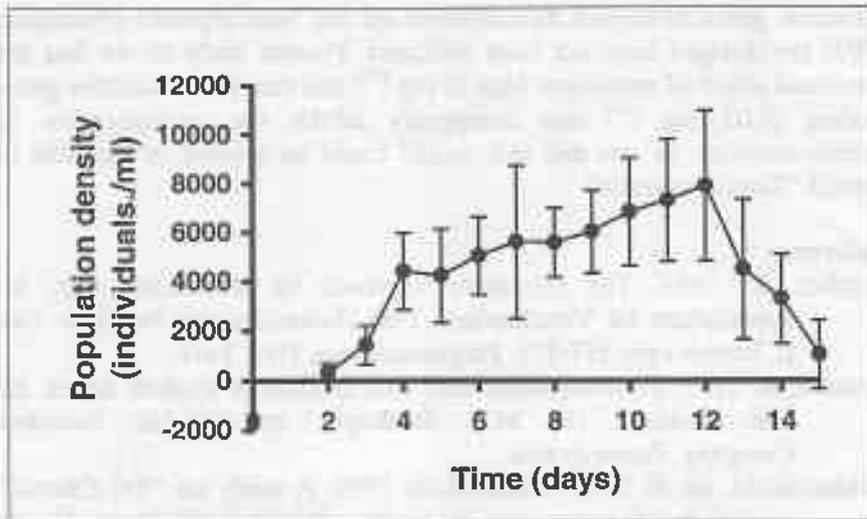
Growth characteristics of *Tetrahymena*.

Figure 3. Growth curve of *Tetrahymena* sp. Vertical bars are \pm S.E.

Short generation time (2.6 h) recorded in the present study revealed that the parasite could multiply in the culture tanks in the presence of dead guppies during a very short period of time. In order to test whether guppy stocks imported are contaminated with this parasite, it could be allowed to multiply in a medium with the best culture conditions found out during the present study. This would be very useful in import risk analysis and even in screening brood stocks transported within the country in order to prevent the spread of the disease. Commonly recommended doses of chloramphenicol given via water for bacterial diseases of fish ranged from $20\text{--}50\ \mu\text{g ml}^{-1}$ (Untergasser 1989; Scott 1993). Significantly lower ($P < 0.01$) population density of *Tetrahymena* sp. recorded in the cultures containing $25\ \mu\text{g ml}^{-1}$ of chloramphenicol suggests that this dosage was not sufficient to inhibit the competing bacterial populations and the maximum inhibition of bacteria occurs at a dose of $50\ \mu\text{g ml}^{-1}$ of the antibiotic. It was observed that chloramphenicol at higher concentrations ($100\ \mu\text{g ml}^{-1}$ and $200\ \mu\text{g ml}^{-1}$) inhibited the *Tetrahymena* population also due to unknown reasons; however, the use of chloramphenicol in aquaculture is totally banned.

The growth curve of clones (Figure 3) obtained shows that *Tetrahymena* sp. could utilize nutrients from dead tissues of guppy over an extended period of time and this is an important fact that should be considered in the control of the spread of this disease.

During the recent outbreak of "Tetrahymenosis", many Sri Lankan guppy farmers used the Leteux Mayer mixture (Scott 1993) without a success, which is a combination of malachite green and formalin, recommended for external protozoan infections. Although the use of

malachite green to control *Tetrahymena* sp. has been reported (Southgate 1993) the dosages have not been indicated. Present study shows that the combined effect of methylene blue (2 mg l^{-1}) and zinc free malachite green oxalate (0.03 mg l^{-1}) can completely inhibit the multiplication of *Tetrahymena* sp. *in situ* and this results could be applied in the field to control "Tetrahymenosis".

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