

IN VITRO EXCYSTATION OF *HAPLORCHIS TAICHUI* (TREMATODA: HETEROPHYIDAE)

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Abstract. The effects of trypsin, bile extract, temperature and acid-based condition for the *in vitro* excystation of *Haplorchis taichui* metacercariae were studied. At 37°C, approximately half the number of metacercariae excysted when exposed to 1% trypsin for 15 minutes with no more excystation found beyond this time. Increasing trypsin concentration seemed to reduce the excystation rate while bile extract was, however, unlikely to be an absolute requirement. A temperature of 37°-41°C yielded a similar excystation result in combination with 1% trypsin; however, less excystation occurred at a lower temperature of 35°C. The acid-based environment of pH 8 gave the best excystation result in association with 1% trypsin at a temperature of 39°C. Higher and lower basicity produced a smaller excystation rate. An environmental condition of 1% trypsin at pH 8 and a temperature of between 37°-41°C was recommended for the *in vitro* excystation of *H. taichui* metacercariae. The relatively broad temperature and pH range condition for the excystation of *H. taichui* corresponded with various definitive hosts that were infected naturally by this fluke.

INTRODUCTION

Haplorchis taichui is a minute intestinal fluke (MIF) that parasitizes the small intestine of birds and mammals including humans (Faust and Nishigori, 1926). This parasite is now recorded as the most common trematode found in the gastrointestinal tract of humans living in northern Thailand (Radomyos *et al.*, 1998). This parasite utilizes fresh water snails (*Melanoides tuberculata*) and fresh water cyprinoid fish as the first and second intermediate hosts, respectively (Malek, 1980). Infections with *H. taichui* in humans are generally acquired by the ingestion of raw and/or undercooked fish harboring a viable infective stage or metacercariae. Clinically, heavy infection of this fluke can cause non-chronic inflammation and segmental enteritis in patients (Sukontason *et al.*, unpublished data).

Several *in vitro* experiments on the excystation of trematodes have been reported (Dixon, 1966; Howell, 1970; Asanji and Williams, 1975; Davies and Symth, 1978; Tielens *et al.*, 1981; Irwin, 1983; Irwin *et al.*, 1993; Chung *et al.*, 1995; El-Mayas and Kearn, 1996; Fried *et al.*, 1997). Little information regarding excystation of MIF is available (Kobayashi *et al.*, 1959; Yasuraoka and Kojima, 1970). Due to the abundance of *H. taichui* in humans living in northern Thailand, such information involving various aspects of this fluke (biology, physiology, ecology, treatment etc) are

needed. Therefore, to increase knowledge on the biology of *H. taichui*, a study on *in vitro* excystation of the metacercariae of this particular fluke was undertaken.

MATERIALS AND METHODS

Metacercariae of *H. taichui* were collected by acid-pepsin digestion of the muscle tissue from cyprinoid fish, *Thynnichthys thynnoides*, caught in Mae Ngud reservoir, Mae Tang district, Chiang Mai Province, northern Thailand. The fish were rinsed using tap water until clean and then dissected. The muscle was digested using acid-pepsin solution [conc hydrochloric acid 1 ml: pepsin (Sigma[®], Germany) 1 g: 0.85% sodium chloride solution 99 ml] in mixer/blender at a ratio of 1 g muscle and 10 ml of acid pepsin solution. The digested material was transferred into a water bath shaker for 1 1/2 hours set at 37°C, then subsequently passed through 2 layers of wet gauze. The digested material was then rinsed with 0.85% sodium chloride solution and examined for metacercariae with a stereomicroscope. The identification of *H. taichui* metacercariae was carried out by morphological examination based on Scholz *et al.* (1991) and sclerites identification on the ventrogenital sac of the metacercariae of *H. taichui* was based on Pearson and Ow-Yang (1982).

Approximately 300 active juvenile metacercariae were selected for the experiments. Since some factors have been reported involving the *in vitro* excystation of trematodes, 4 important factors have been chosen

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and consecutively determined for the excystation, namely trypsin, bile extract, temperature and pH.

The effect of 1%, 5% and 10% trypsin (Sigma[®], Germany) on the *in vitro* excystation was tested initially. Since 37°C is the normal human body temperature, it was set in a water bath shaker for trypsin assessment in the first experiment. The number of excysted metacercariae was counted every 15-minutes for 1½ hours using a stereo-microscope. The excystation was scored when a juvenile had escaped completely from the ruptured cyst wall. Secondly, the effect of bile extract was determined. Bile extract was obtained from the Sigma Chemical Company (City) and concentrations of 1%, 5% and 10% were tested. Thirdly, the effect of temperature on excystation was tested. The temperature in a water bath shaker was set at 35°, 37°, 39° and 41°C. Lastly, the effect of an acid-base environment was determined by incubating 30 metacercariae at pH 5, 7, 8, 9, 10 and 13 in combination with the most suitable trypsin and temperature obtained from previous experiments. The osmolarity of respective buffers was adjusted using NaCl solution. All experiments assessed for each factor were performed in duplicate.

Data were analyzed by using the SPSS program version 7.5 for Windows. The chi-square test was employed to determine whether the number of excysted worms differed significantly within the same condition used. A p-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Excysted *H. taichui* metacercariae obtained from fish were usually slightly yellow in color and appeared to be spherical. When cysts were placed in an excystation medium set at 37°C, metacercariae became active within 15 minutes. Excystation events were similar to those of Fried *et al* (1997) in that the organism rotated within the cyst (activation), followed by a breaching of the cyst before the release of the organism from all cyst walls. Excystation occurred when the outer cyst was disrupted and the organism was free in the medium. In the first experiment, excystation started 15 minutes before there was a presence of 1%, 5% and 10% trypsin, but a maximum of 53.3% was reached at 1% trypsin (Fig 1). However, there was no significant difference in excystation rate among 1%, 5% and 10% trypsin ($p=0.164$; chi-square test).

Fig 2 shows the effect of bile extract on excystation. It was found not to be essential for *H. taichui* excystation, as indicated by the very low rate in all 1%, 5% and 10% bile extract supplements.

The effect of temperature on excystation with the presence of 1% trypsin is shown in Fig 3. No significant difference in percentage excystation was found at 37°, 39° and 41°C (50.0-56.7%). All metacercariae excysted in less than 15 minutes. Although the percentage of excystation was lower when cysts were treated at 35°C, it was not significantly different from that at 37°, 39° and 41°C ($p=0.4$). Accordingly, a temperature of 39°C was chosen to be an integral part of the pH activation stimulus.

The effect of an acid-base environment (pH) was also monitored and the result is shown in Fig 4. In experiments set at 39°C with the presence of 1% trypsin, the excystation was highest at pH=8 within 15 minutes (73.3%) and increased to 83.3% in 90 minutes with no statistical difference. Nevertheless, there was no statistically significant difference among excystations at pH 5-9 ($p>0.05$), with a lower rate yielded at the beginning of pH 10 and no excystation occurred at pH 13.

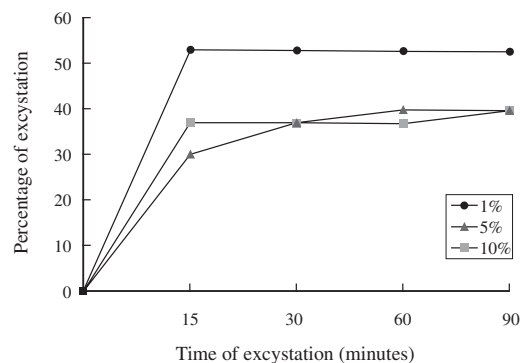


Fig 1- Excystation of *Haplorchis taichui* metacercariae in varying trypsin concentrations (1%, 5% and 10%) at a temperature set at 37°C.

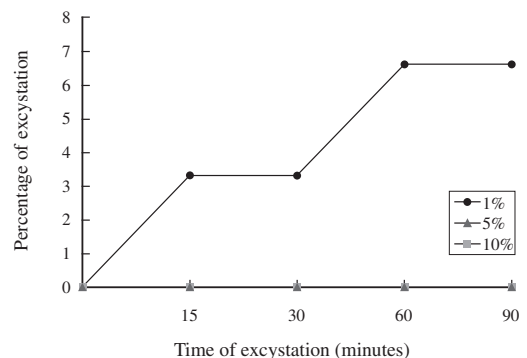


Fig 2- Excystation of *Haplorchis taichui* metacercariae in varying bile extract concentrations (1%, 5% and 10%) at a temperature set at 37°C.

Different fluke species utilize different extrinsic and intrinsic factors of the digestive tract in a definitive host, thus, triggering excystation of metacercariae (Chung *et al*, 1995). Trypsin was reported to be one absolute requirement for the excystation of the fluke, *Echinoparyphium serratum*, and presumably it weakened the capsule sufficiently for the vigorous activity of the metacercariae to cause the fluke to rupture (Howell, 1970). In some cases, pretreatment with acid pepsin was a definite prerequisite to trypsin solution for excystation (Lackie, 1975), but in others it was not (El-Mayas and Kearn, 1996). Erasmus and Bennett (1965) demonstrated that acid pepsin pretreatment speeded up the excystation of *Cyathocotyle bushiensis*, but it was not a prerequisite. Similarly, pretreatment of *E. serratum* and *Zygoctyle lunata* cysts with pepsin also resulted in faster excystation rates (Howell, 1970; Irwin *et al*, 1993),

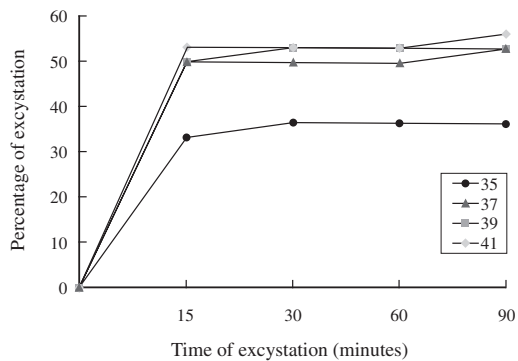


Fig 3- Excystation of *Haplorchis taichui* metacercariae in varying temperatures (35°, 37°, 39° and 41°C) with the presence of 1% trypsin solution.

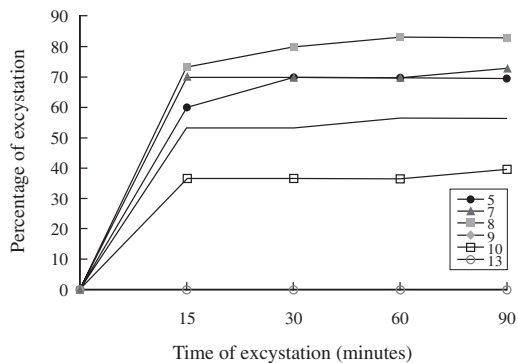


Fig 4- Excystation of *Haplorchis taichui* metacercariae in varying acid-base environments (pH=5, 7, 8, 9, 10 and 13) with the presence of 1% trypsin solution and a temperature set at 39°C.

but it was not essential for a high percentage of excystation to take place (Howell, 1970). Pepsin and trypsin facilitated the excystation of *Bucephalus haimeanus*, but they were not essential since some of the active metacercariae perforated the cyst wall in their absence (El-Mayas and Kearn, 1996). Besides these factors, as in the case of pepsin or trypsin, the study of Chung *et al* (1995) has shown that an intrinsic factor such as cysteine proteases secreted by *Paragonimus westermani* metacercariae modulate excystation.

An experiment on the excystation of *H. taichui* has shown that bile extract is unlikely to be an absolute requirement. This result is in accordance with the excystation of *Maritrema arenaria* (Irwin, 1983) and *P. westermani* (Chung *et al*, 1995), but in discordance with *Metagonimus yokogawai* (Kobayashi *et al*, 1959) and *Fasciola hepatica* (Dixon, 1966). According to Asanji and Williams (1975), bile was found not to be an absolute necessity for the excystation of *Parorchis acanthus*, *Posthodiplostomoides leonensis*, *Posthodiplostomum* sp and *Clinostomum tilapiae*, but its presence in the medium increased the rate of excystation. Juvenile *F. hepatica* penetrated the gut wall more rapidly in the presence of bile than in its absence (Tielens *et al*, 1981). Bile salt has been claimed to possibly act by increasing the effect of enzymes secreted by the metacercaria and induce muscular movements in the metacercaria (Dixon, 1966), or it might cause permeability changes in the membranes of cysts (Lackie, 1975).

Regarding the temperature and pH, these factors show a relatively broad range used for the *in vitro* excystation of *H. taichui*. The temperature of 39°C in this study was the same as that used in *F. hepatica* or *E. serratum* excystment (Dixon, 1966; Howell, 1970). The experiment of Howell (1970) suggested that an elevated temperature had been required for excystation to occur.

In conclusion, the factors favoring *in vitro* excystation of *H. taichui* metacercariae were 1% trypsin, a temperature of 37°-41°C and a pH of 5-8. The relatively broad temperature and pH range requirements for excystation may be one of the explanations why various definitive hosts can be parasitized naturally by *H. taichui*.

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