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

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Abstract. Newly excysted metacercariae of *Haplorchis taichui* were cultured in a candle jar set at 37 ˚C. Both monophasic culture media [0.85% NaCl, RPMI 1640, RPMI 1640+10% fetal calf serum (FCS)] and diphasic culture media [RPMI 1640 + egg yolk agar, RPMI 1640 + 5%, 10% or 15% blood in blood agar (BA), RMPI 1640 + 5%, 10% and 15% FCS with 5% blood in BA] were used *in vitro*. Parasites survived for only 1 day in 0.85% NaCl without any development. In RPMI 1640 with egg yolk agar and RMPI 1640 + 5%, 10% FCS, the parasite survived for 3-5 days. In contrast, worms survived for 12-14 days in RPMI 1640 with blood agar without any change in result in a different concentration of blood in BA. The ovary and testes were observed after 3 days incubation in this media. Nevertheless, only 1 parasite in RPMI 1640 with 15 % blood in BA had vitellaria and eggs at day 6. RPMI 1640 with blood agar can be used as short-term maintenance for the *in vitro* culture of *H. taichui*. However, further studies are needed.

INTRODUCTION

In vitro cultivation of parasitic helminths, including digenetic trematodes, has been a valuable tool in medical parasitology, permitting and/or facilitating the development of diagnostic reagents, chemotherapeutic agents and vaccines, and providing insights into naturally complex host-parasite interactions (Coustau and Yoshino, 2000). It would provide parasites in large numbers at a low cost and in a relatively defined medium in which studies of their biology, physiology, behavior, etc could be made with comparative ease (Irwin, 1997).

Haplorchis taichui is the most common trematode that parasitizes human populations in northern Thailand (Pungpak *et al*, 1998; Radomyos *et al*, 1998). It is a minute intestinal fluke in several species of birds and mammals serving as the definitive host, and many cyprinoid fish species being the second intermediate host (Faust and Nishigori, 1926; Srisawangwong *et al*, 1997; Sukontason *et al*, 1999). This suggests its broad range in host specificity. The prevailing occurrence of *H. taichui* has led to studies in various aspects of this fluke in order to gain knowledge that could provide strategies in prevention, control, and the development of an antihelminthic or vaccine against this parasite. Thus, the objective of this study was to assess the *in vitro* development of *H. taichui* under a controlled condition.

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MATERIALS AND METHODS

Metacercariae and excystment

Metacercariae of *H. taichui* were isolated from cyprinoid fish (*Thynnichthys thynnoides*) collected from Mae Ngud Somboonchol Dam, Mae Tang district, Chiang Mai Province of northern Thailand. Metacercariae were collected by acid pepsin digestion of the fish tissues (Sukontason *et al*, 2000). The excystment of metacercariae was carried out using the condition of 1% trypsin (Sigma®), pH 8 at 39˚C for 15 minutes in a water bath shaker. The active metacercariae were selected for *in vitro* development.

Culture media

Every step of the procedure was performed aseptically. The culture media used in this study were developed from methods used in the cultivation of *Fasciola hepatica* (Smith and Clegg, 1981) and *Paragonimus westermani* (Kannangara, 1974). After being washed 3 times with 0.85% sterile NaCl, the metacercariae were repeatedly washed 2 times with RPMI 1640 (Sigma®) containing 200 units/ml of penicillin, 100 μg/ml of gentamicin, 20 mM HEPES buffer and 26.7 ml of sodium bicarbonate solution (7.5% w/v), pH 7.4. Fifty metacercariae were introduced aseptically into a petri dish (35 mm in diameter) containing each culture medium, and were incubated with 5-10% CO_2 at 37˚C. The experiments were duplicated.

The components of the different culture media used are shown in Table 1. RPMI 1640 (Sigma®) was used as a core component for each medium. The fetal

calf serum [FCS; (Sigma®)] was made up as 1%, 5% and 10% in RPMI 1640. The egg-yolk agar was freshly prepared using newly laid chicken eggs. Five to ten percent of egg yolk concentration was mixed in 0.7% agar (nutrient agar; DIFCO), which had been sterilized before use by autoclaving at 121˚C, with a pressure of 15 lb/inch^2 for 15 minutes. The blood agar (BA) was prepared using human group O blood from the blood bank of Maharaj Nakhon Chiang Mai Hospital. It was made up as 5%, 10% and 15% blood in BA and the preparation was made in the same manner as the egg - yolk agar.

Culture media were categorized into monophasic culture medium and diphasic culture medium (Table 1). Two milliliters of media A, B and C were placed in to a sterile plastic petri dish (diameter 35 mm; Nunc). As for media D,E,F,G,H,I and J, the lower part was solid medium and contained 1.5 ml of agar, while the upper part comprised 1.5 ml of liquid medium containing 200 units/ml of penicillin and 100 μg/ml of gentamicin, which had been sterilized by filtration with a 0.2 mm millipore filter (MINISART) before use. The medium was adjusted to pH 7.4 using 7.5% (w/v) $NaHCO_3$. The liquid medium was changed daily to prevent contamination. The assessment of *in vitro* development was observed daily using a stereo-microscope at 10 x and 40 x. Some worms were randomly selected at intervals of 3 days. They were flattened on a glass slide with a cover slip, fixed with alcohol acetic acid, stained with Mayer's carmine, mounted with Permount® (Fisher Chemical) and examined using a conpound microscope at 100 x and 400 x. The survival rate and development of the genital rudiment of the worms were assessed in each *in vitro* experiment.

RESULTS

The survival rate of *H. taichui* under each *in vitro* experiment is shown in Fig 1. Parasites survived for only 1 day in 0.85% NaCl without any further development. In media B (RPMI 1640), C (RPMI 1640 + 10% FCS) and D (RPMI 1640 + egg yolk agar), the parasites survived for only 3-5 days. In contrast, they survived for 12-14 days in the diphasic media of E, F, G, H, I and J. The ovary and testes were observed after 3 days of incubation in this diphasic media (Fig 2). The eggs and vitellaria were seen in the uteri of one worm that was cultivated at day 6 in medium G (RPMI 1640 + 15 % blood in BA) (Fig 3).

DISCUSSION

Cultivation of trematodes through a substantial part of the life cycle is essential for determining the precise nutritional requirements of the parasite (Clegg and Smith, 1987). Success of *in vitro* cultivation has been judged in terms of the longevity of the cultures, growth of the trematodes, and the rate and extent of the development of the organisms (Irwin, 1997). In this study, the control media of only RPMI 1640 (medium B), media supplemented with only 10% FCS (medium C) or egg - yolk agar (medium D) showed no distinction on the development of *H. taichui*. A similar effect of egg yolk supplement was obtained for another minute intestinal fluke, *Metagonimus yokogawai* (Yasuraoka

Table 1
Component of media used for *in vitro* cultivation of *Haplorchis taichui*.

Components of culture media	Monophasic media			Diphasic media						
	A	B	C	D	E	F	G	H	I	J
0.85% NaCl	√									
RPMI 1640		√	√	√	√	√	√	√	√	√
5% Fetal calf serum								√		
10% Fetal calf serum			√						√	
15% Fetal calf serum										√
Egg yolk agar				√						
5% Blood in blood agar					√			√	√	√
10% Blood in blood agar						√				
15% Blood in blood agar							√			

√ indicate the components in each culture media.

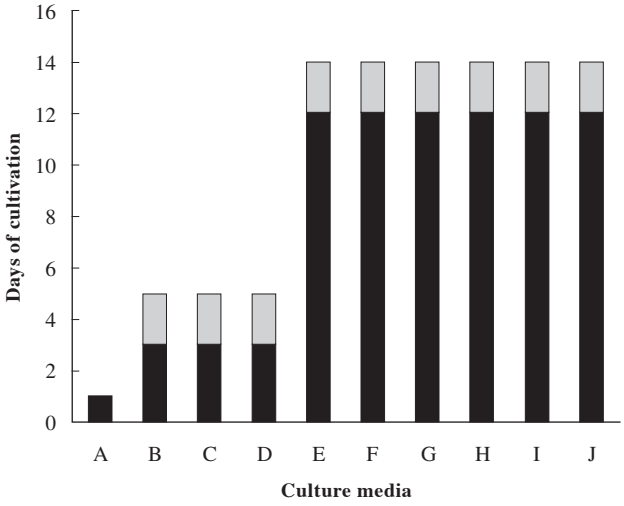
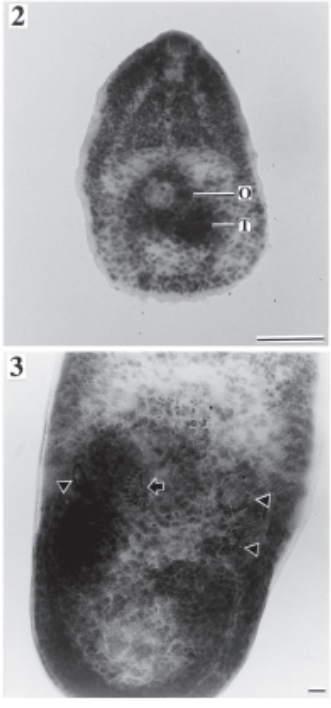


Fig 1- Longevity of adult *Haplorchis taichui in vitro* cultured in different media. Monophasic media were A-C, while diphasic media were D-J. Dark bars indicate time when most worms were alive.



Figs 2,3- Light micrographs of the *in vitro* development of *Haplorchis taichui* stained with Mayer's carmine. (2) Three-day-old worm cultured in diphasic medium with the solid phase containing blood agar. Ovary (O) and testis (T) appeared. Bar = 50 μm. (3) Six-day-old worm cultured in medium G with the solid phase containing 15% blood in blood agar. Arrow and triangles indicate ventrogenital sac and eggs, respectively. Bar = 10 μm.

and Kojima, 1970) and the lung flukes, *P. westermani* (Yokogawa *et al*, 1955), *Paragonimus miyazakii* and *Paragonimus ohirai* (Hata *et al*, 1987), but was in contrast to *P. westermani* (Kannangara, 1974).

Various media have been used for the *in vitro* cultivation of the helminthic parasite. No significant difference among RPMI 1640, Eagle's medium and NCTC 109 was noted (Kook *et al*, 1997). In this study, the supplement with only RPMI 1640 (medium B) or RPMI 1640 + egg yolk agar (medium D) showed no difference in the 3-5 day survival rate of *H. taichui*. Only the cultivation of *M. yokogawai* in NCTC 109 resembled this result (Yasuraoka and Kojima, 1970). Nonetheless, the NCTC 109 or NCTC 135 medium, supplemented with egg yolk, was the basic requirement for the earlier development of *Fibricola seoulensis* fluke (Seo, 1989).

No marked difference of *H. taichui* survival rate in media E-J indicated that serum is needed as a nutrient for the survival and maintenance of this intestinal worm. A similar finding was observed by Kook *et al* (1997) for *Gymnophalloides seoi*, and these authors also indicated that the sera did not seem essential for the sexual maturation of this intestinal fluke. The role of red blood cells (RBC), supplemented in medium for enhancing the development of worms, has been documented by *P. westermani* (Yakogawa *et al*, 1955), *M. yokogawai* (Yasuraoka and Kojima, 1970) and *P. ohirai* (Hata *et al*, 1987). Hemoglobin acts as an important nutrient in this regard (Hieb *et al*, 1970; Zussman *et al*, 1970). The ingestion of RBC from the

culture media was reported in *P. miyazakii* (Hata *et al*, 1987). The presence of RBC inside the digestive tract of *H. taichui*, observed in the biopsy specimen of the small intestine of a patient, corresponds with this finding (K Sukontason, unpublished data). In the present study, even one worm cultured in medium G (RPMI 1640 + 15% blood in BA) possessed eggs and vitellaria, which providing evidence that this medium containing blood could be, at least, used to enable *in vitro H. taichui* to be ovigerous. This investigation merits further studies.

The temperature of the medium is also an important factor for the *in vitro* cultivation of trematodes (Irwin, 1997). In this study, a temperature of 37˚C similar to human body temperature was used. The intestinal worm, *G. seoi*, developed *in vitro* better at 41˚C than 37˚C (Kook *et al*, 1997). However, it is apparent that bird parasites develop best at 42˚C, whereas those of mammals seem to prefer 37˚C (Irwin, 1997). Since *H. taichui* can utilize birds and/or mammals as the definitive host (Faust and Nishigori, 1926), the partial success for *in vitro* cultivated parasites in this study possibly requires a higher temperature of 41˚-42˚C.

It is generally known that the growth and development of parasitic helminths *in vitro* are slower and smaller in size than those developed *in vivo* (Taylor and Baker, 1987; Irwin, 1997; Kook *et al*, 1997). Also, it has taken at least 2-3 times longer *in vitro* than *in vivo* to develop adult flukes (Yasuraoka and Kojima, 1970). The *in ovo* cultivation using the chorioallantoic membrane (CAM) has been reviewed by Irwin (1997). It is likely that the worm development of *in ovo* is required for *in vitro* and *in vivo* (Fried and Rosa-Brunet, 1991). Irwin (1997), however, suggested that the advantages of *in vitro* cultivation over *in vivo* and *in ovo* techniques for the maintenance and development of trematodes are obvious. As for *H. taichui*, the short-term maintenance for *in vitro* cultures could be made to provide organisms in such biological studies. However, further studies are still needed to achieve greater success.

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REFERENCES

Clegg JA, Smith MA. Trematoda. In: Taylor AER, Baker JR, eds. *In vitro* methods for parasite cultivation. London: Academic Press, 1987:254-81.

Coustau C, Yoshino TP. Flukes without snails: advances in the *in vitro* cultivation of intra-molluscan stages of trematodes. *Exp Parasitol* 2000;94:62-6.

Faust EC, Nishigori M. The life cycles of two new species of Heterophyidae, parasitic in mammals and birds. *J Parasitol* 1926;13:91-132.

Fried B, Rosa-Brunet LC. Cultivation of excysted metacercariae of *Echinostoma caproni* (Trematoda) to ovigerous adults on the chick chorioallantois. *J Parasitol* 1991;77:568-71.

Hata H, Yokogawa M, Kobayashi M, Niimura M, Kojima S. *In vitro* cultivation of *Paragonimus miyazakii* and *P. ohirai*. *J Parasitol* 1987;73:792-6.

Hieb WF, Stokstad ELR, Rothstein M. Heme requirement for reproduction of a free-living nematode. *Science* 1970;168:143-4.

Irwin SWB. Excystation and cultivation of trematodes. In: Fried B, Graczyk TK, eds. Advances in trematode biology. Florida: CDC Press, 1997:57-86.

Kannangara DW. *In vitro* cultivation of the metacercariae of the human lung fluke *Paragonimus westermani*. *Int J Parasitol* 1974;4:675-6.

Kook J, Lee SH, Chai JY. *In vitro* cultivation of *Gymnophalloides seoi* metacercariae (Digenea: Gymnophallidae). *Korean J Parasitol* 1997;35:25-9.

Pungpak S, Radomyos P, Radomyos B, Schelp FP, Jongsuksuntikul P, Bunnag D. Treatment of *Opisthorchis viverrini* and intestinal fluke infections with praziquentel. *Southeast Asian J Trop Med Public Health* 1998;29:246-9.

Radomyos B, Wongsaroj T, Wilairatana P, *et al*. Opisthorchiasis and intestinal fluke infections in northern Thailand. *Southeast Asian J Trop Med Public Health* 1998;29:123-7.

Seo BS. Comparative growth and development of the metacercariae of *Fibricola seoulensis* (Trematoda: Diplostomidae) *in vitro*, *in vivo* and on the chick chorioallantois. *Kisaengchunghak Chapchi* 1989;27:23-48.

Smith MA, Clegg JA. Improved culture of *Fasciola hepatica in vitro*. *Z Parasitenkd* 1981;66:9-15.

Srisawangwong T, Sithithaworn P, Tesana S. Metacercariae isolated from cyprinoid fishes in Khon Kaen district by digestion technic. *Southeast*

Asian J Trop Med Public Health 1997;28 (suppl): 224-6.

Sukontason K, Piangjai S, Muangyimpong Y, Sukontason K, Methanitikorn R, Chaithong U. Prevalence of trematode metacarcariae in cyprinoid fish of Ban Pao district, Chiang Mai Province, northern Thailand. *Southeast Asian J Trop Med Public Health* 1999;30:365-70.

Sukontason K, Sukontason K, Muangyimpong Y, Piangjai S. Treatment of *Haplorchis taichui* in *Mus musculus* mice. *Exp Parasitol* 2000;94:48-50.

Taylor AER, Baker JR. *In vitro* methods for parasite cultivation. London: Academic Press, 1987.

Yasuraoka K, Kojima K. *In vitro* cultivation of the heterophyid trematode, *Metagonimus yokogawai*, from the metacercariae to adult. *Jpn J Med Sci Biol* 1970;23:199-210.

Yokogawa M, Oshima T, Kihata M. Studies to maintain excysted metacercariae of *Paragonimus westermani in vitro*. *Jpn J Parasitol* 1955;4:70-5.

Zussman RA, Bauman PM, Petruska JC. The role of ingested hemoglobin in the nutrition of *Schistosoma mansoni*. *J Parasitol* 1970;56:75-9.