***In Vitro* Cultivation of Newly Excysted Metacercariae of Japanese *Fasciola* sp.**

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A number of attempts have been made to cultivate *Fasciola* spp. Physiological saline and Ringer solution were initially used to maintain flukes recovered from infected animals, but fluke survival times were considerably short [14, 15]. Subsequently, Hédon-Fleig solution, Earle's balanced salt solution, and Hanks' balanced salt solution were used with some nutritional ingredients to cultivate *Fasciola hepatica* flukes, at stages from newly excysted metacercariae to adult flukes [3, 6, 16]. In those studies, the flukes were maintained for a few days, although they did not increase in size.

Recently, tissue culture methods have been adopted for the cultivation of *F. hepatica* and *F. gigantica* [2, 7, 9, 13]. Smith and Clegg [13] attempted to cultivate newly excysted metacercariae of *F. hepatica* using RPMI 1640 medium supplemented with 50% human serum and 2% human red blood cells, and found that the flukes increased in size to a maximum length of 7 mm and could be maintained for a maximum period of 98 days. Hanna *et al.* [7] cultivated newly excysted metacercariae of *F. gigantica* using Eagle's MEM medium supplemented with 20% fetal calf serum and fetal calf spleen cells for over 60 days.

In vitro cultivation of *Fasciola* flukes has been used in immunological, pathological and physiological studies on *Fasciola* and fascioliasis [1, 4, 5, 10, 11].

This paper deals with the cultivation of newly excysted metacercariae of Japanese *Fasciola* sp.

Metacercariae were released from their cysts by the method described previously [8]. The number of newly excysted metacercariae used in each trial ranged from 19 to 58, measuring $250 \times 140 \mu\text{m}$ in mean length and breadth, respectively. Basal media used were RPMI 1640 (Nissui Pharmaceutical Co., Tokyo), Eagle's MEM (Nissui) and Medium 199 (Nissui), with supplementation of $300 \mu\text{g/ml}$ L-glutamine to the former two media. Unless otherwise stated, 50% fetal calf serum, 0.2% NaHCO_3 , 200 units/ml penicillin

and $200 \mu\text{g/ml}$ streptomycin were added to each medium. As test ingredients, 5 and 10% liver infusion, 2% fructose, and 2% sheep red blood cells were used. The liver infusion was prepared from the Liver Descicated (Oxoid, U. K.) according to the directions supplied. Before cultivation, newly excysted metacercariae were washed with saline containing 1,000 units/ml penicillin and 1 mg/ml streptomycin prewarmed at 37°C . For cultivation, about a dozen excysted metacercariae were placed in a Petri dish 70 mm in diameter, which contained 5 ml of test medium. Then the metacercariae were cultivated at 37°C under an atmosphere of 5% CO_2 in air. The medium was replaced at 3–4-day intervals. All procedures were done aseptically. Survival of excysted metacercariae was judged by appearance and motility at 4- or 7-day intervals.

Newly excysted metacercariae of *Fasciola* sp. were cultivated in RPMI 1640, Eagle's MEM and Medium 199, each supplemented with 50% fetal calf serum (Figs. 1 and 2). Metacercariae cultivated in RPMI 1640 survived for 4–40 days. The survival rate was considerably high up to the 12th day of cultivation ($79.2\% \pm$), and gradually decreased thereafter. Mean survival time was 16.3 days. The body size of cultivated metacercariae increased very slightly, as shown in Fig. 2. Development of the inner organs was not recognized (Fig. 3). The survival rate of metacercariae cultivated in Eagle's MEM was lower than that for RPMI 1640. The maximum and mean survival times were 24 and 11.5 days, respectively. As for Medium 199, cultivated metacercariae died faster than those cultivated in RPMI 1640 or Eagle's MEM. The survival rate fell to 21.7% by the 8th day of cultivation, and the maximum and mean survival times were 16 and 6.2 days, respectively.

Some ingredients were tested by addition to RPMI 1640 or Eagle's MEM to evaluate their efficacy on the survival of excysted metacercariae (Table 1). However, none of the ingredients was effective for increasing the survival rate.

The results obtained in the present study are not directly comparable with those previously

reported for *F. hepatica* [2, 13] or *F. gigantica* [7] on account of the differences in *Fasciola* species, basal medium and serum used. However, it is considered that newly excysted metacercariae of

Fig. 1. Survival of newly excysted metacercariae of *Fasciola* sp. cultivated in media: O, RPMI 1640; ●, Eagle's MEM; □, Medium 199. All media were supplemented with 50% fetal calf serum.

Fig. 2. Growth in body length of excysted metacercariae cultivated in RPMI 1640 (O), Eagle's MEM (●), or Medium 199 (□).

Japanese *Fasciola* sp. were successfully cultivated for a considerably long period by using RPMI 1640 supplemented with fetal calf serum in the present study.

Addition of red blood cells [2, 13], glucose [12], fructose [14] or liver extract [12] has been reported to be effective for the survival of *Fasciola* spp. since the early period of cultivation studies, although this has not been unequivocally confirmed in more recent investigations. These ingredients showed no remarkable effects in the present study, and therefore they might not be important factors for the survival or growth of excysted metacercariae of *Fasciola* sp.

No remarkable growth in body size nor development of the inner organs of the cultivated juvenile flukes was observed in the present study, as in previous investigations. Thus, further studies will be needed on the physiology of *Fasciola* sp., such as nutrition, gas phase and so on, in

Fig. 3. Growth of excysted metacercariae cultivated in RPMI 1640. 0, newly excysted; 1, cultivated for 1 week; 2, cultivated for 2 weeks; 3, cultivated for 3 weeks.

Table 1. Effect of ingredients on survival of excysted metacercariae

Ingredients	No. of excysted metacercariae tested	Survival time (days)	
		Mean	Range
Glucose 1%	48	12.4	<7-21
Fructose 2%	24	8.5	<4-28
Liver infusion			
5%	19	6.3	<4-16
10%	56	12.3	<7-21
Sheep red blood cells 2%	25	8.6	<4-32

a) Glucose, fructose, and liver infusion were used in RPMI 1640 while sheep red blood cells were used in Eagle's MEM. All media were supplemented with 50% fetal calf serum.

order to cultivate newly excysted metacercariae with success, which closely resemble those occur normally in the liver.

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要 約

日本産脱囊幼肝蛭の人工培養（短報）：河野潤一・石丸 司・清水 晃・木村 重（神戸大学農学部家畜衛生学教室）——日本産肝蛭メタセルカリアから人工的に脱囊させた幼肝蛭を、それぞれ50%牛胎児血清を添加したRPMI 1640, Eagle's MEM および Medium 199の3種の培地を用い、37°C, 5% CO₂ 下で培養した。幼肝蛭はRPMI 1640を基礎培地としたもので最も良く培養され、培養12日における生存率は79.2%と高率であり、平均生存日数は16.3日、最長40日まで生存した。