Original Article

Maintenance of Liver Fluke, *Dicrocoelium dendriticum*, Outside the Body of its Native Host

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Abstract

Aims: *In vitro* cultivation of digenea would help the development of effective treatments and studies of the biology of the parasites. The goal of the present study was to optimize culture conditions for the maintenance liver fluke, *Dicrocoelium dendriticum*. **Materials and Methods:** Forty fresh *D. dendriticum* were collected from the sheep liver and washed three times with warm Roswell Park Memorial Institute (RPMI) 1640 Medium. The collected worms were transferred to 24-well Nunc-Immuno plates containing RPMI media supplemented with 50% of fetal bovine serum (FBS), 2% of sheep red blood cells (RBCs), 50 IU/ml of penicillin, and 50 mg/ml streptomycin. The mobility of the live/dead worms was observed by inverted microscope. The mean and median survival time was calculated by Kaplan–Meier model, and survival and hazard function graphs were also analyzed. **Results:** *D. dendriticum* was lived *in vitro* only for long periods of about 25 dyes. The 1st day of maintaining in culture media, one worm was dead and the number of dead worms was raised to 40 after 25 days of incubation. On the one hand, the mean survival time was 392 h with a confidence interval (CI) of 95% (384.8–400.03). On the other hand, the median survival time was 420 h with a CI of 95% (406.9–433.09). *D. dendriticum* was able to be alive in RPMI 1640 media for at least 25 days. **Conclusion:** RPMI 1640 supplemented with FBS, and RBCs can be used as short-term maintenance for the *in vitro* culture of *D. dendriticum*. The outcomes of the current study could be useful for many aspects of parasitological analysis.

Keywords: Culture media, *Dicrocoelium dendriticum*, *in vitro*, Roswell Park Memorial Institute medium, survival rate

Introduction

Helminths are the most common parasites infecting humans as well as animals in all countries. It is estimated that almost 2 billion people worldwide are infected with helminths. Helminths are transmitted to humans through food, soil, arthropod, and molluscan vectors. They are found in the intestines, liver, lungs, blood, and occasionally, the brain and other organs. There are two major groups of parasitic helminths including nematodes and flatworms.[1] Members of the trematoda group (flatworms) are important infectious helminths for humans as well as animals. As an illustration, the family of *Dicrocoeliidae* (members of the Trematoda group) includes important human pathogens of the genus *Dicrocoelium*. *Dicrocoelium dendriticum*, known as a common parasite of the liver, commonly lives in the bile duct and gall bladder of different domestic and wild animals, especially ruminants and occasionally humans. Living *D. dendriticum* parasite in the liver of animals has many destructive effects including decreasing the production of meat, milk, and wool in the entire world and consequently economic collapse.[2,3] At present, this small liver fluke counts as the major problem in northern and southern Europe, northern Africa, western and eastern America, and Asia. Besides, this lancet fluke commonly exists within most Mediterranean countries thanks to the appropriate

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biological conditions.[3-5] Moreover, among Asian countries, Iran has been known as one of the most endemic regions of this infection, and annually highest reports are recorded from the various provinces of Iran.[3,6] The capability to culture trematode outside their host, especially *in vitro* statue, is fundamental for many aspects of parasitological purpose. In fact, *in vitro* culture provides a great opportunity to easily change and control experimental conditions to reach optimum conditions.[7] A wide variety of information related to the ability to grow the life stages of digenean trematodes *in vitro* conditions would benefit the development of nutritional necessities, anthelmintic drugs, and effective vaccines as well as facilitate research into the epidemiological pattern, ecology, evolution, genetics, behavior, metabolism, physiology, and host-parasite of these worms.[8-10] Some researchers have been done in which they successfully cultured the larvae stages of the little parasitic worm and have introduced the primary axenic culture system for long *in vitro* maintenance of larvae.[11,14] However, no information was published on the ability of trematode species *in vitro* culture to grow from egg stage to a developed adult ready to reproduce. It is worth mentioning that it is not possible to completely replace *in vitro* culture with the definitive host. The one thing is that cultivation processes of helminths are quite difficult in comparison with protozoa microorganisms due to the complexity of body configuration, metabolism, and life cycles with multiple hosts, especially in the case of trematodes.[15] The other thing is that different parasites need different cultivation conditions such as temperature, nutrients, and incubation conditions. In most of the helminths, parasitic infections culture could not be a routine identification technique. However, culture helps clinch the diagnosing in several parasitic infections and it has a great function in research relating to pathogenic parasites.[15-18] Due to the lack of enough knowledge related to all stages life cycle of liver flukes throughout their *in vitro* cultivation processes, the goal of the present study was to optimize culture conditions for the maintenance of trematode, *D. dendriticum*.

Materials and Methods

A total of 40 fresh adult *D. dendriticum* were collected through necropsy of livers, bile ducts, and gallbladder of sheep slaughtered from a local abattoir in Kashan city, Iran. Then, collected worms were washed three times with a warm sterile Roswell Park Memorial Institute (RPMI) 1640 culture medium. Afterward, samples were identified morphologically based on standard keys.[19]

In this study, the culture media were developed based on the utilized strategy in the cultivation of *Haplorchis taichui* (Trematoda: *Heterophyidae*).[20] To *in vitro* evaluation, at first, the collected worms were washed three times with Phosphate-buffered saline (PBS) (0.85%) and then were washed two times with warm RPMI 1640 Medium with L-glutamine and sodium bicarbonate (Sigma Aldrich CO, USA). In the next step, worms were transferred into 24-well Nunc-Immuno plates containing RPMI 1640 culture medium supplemented by fetal bovine serum (FBS) 50% (v/v), sheep red blood cells (RBCs) 2% (v/v), penicillin (50 IU/ml), and streptomycin (50 mg/ml). Moreover, it needs to be mentioned that various concentrations of the media supplements such as RBC and FBS were tested. Afterward, the plate was incubated in a CO_2 incubator at 5% atmosphere. Finally, the numbers of live parasites per well were considered as quantify of culture success for up to 25 days. For full credibility of the study, a control group created and the experiment was performed in three times replicates in similar CO_2 conditions in 0.9 NaCl. All steps of the procedures were undertaken under a sterile laminar flow cabinet. Figure 1 shows the comparison between the dead and alive worm by staining with eosin (0.1%). Based on Figure 1, a dead worm has red or pink color which is due to the absorption of eosin. On the other hand, the live worm remains colorless and exhibited suckers activity and movement. Vital stain, as one of the diagnostic techniques, is used for staining living organisms for microscopical observation. In this technique, viability can be assessed by counting the proportion of unstained and stained cells. To stain cells, 1 g of eosin dye was dissolved in 1 L of distilled water to make eosin solution (0.1%). Then, 200 µL of the above dye was added to collected worms in a microtube. Afterward, one worm was separated from the microtube onto a microscope slide and cover with a 24 mm × 50 mm cover glass. Finally, unstained and stained worms were considered viable and nonviable 20 min after staining, respectively. In this study, the motility of the parasite and its reaction to eosin staining were considered as the index of survival rate. In all experiments, after staining, the survival of the adult worms was confirmed after 30 s exposed to the culture medium. Consequently, the motility of the live or dead worms was evaluated under an inverted microscope at 4× within several days in each 12 h. As a result, under 5% CO_2 atmosphere, the maximum survival was 25 days. In all experiments, the survival rate of adult worms was investigated each 12 h for 25 days, which was including 30 s observation under a microscope. As a result, the number of viable worms was a sign of successful culture. The mean and median survival time of worms were calculated by Kaplan–Meier model. In addition, survival and hazard function graphs of worms were drawn.

Results

In this study, 40 fresh adult *D. dendriticum* were studied at interval times 24 h. After the first 24 h, one worm was dead and 39 worms were alive. Over time, the number of dead worms was increased so that the number of worms after the 25th cultivation was zero [Table 1]. The mean survival time for

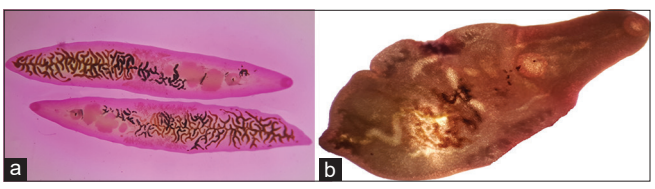

Figure 1: Comparison between (a) dead and (b) alive worm

Table 1: Rate of alive worms over different time incubation according to Eosin reaction

Day	12 h, *n* (%)	24 h, *n* (%)
First	40 (100)	39 (97.5)
Second	37 (92.5)	35 (87.5)
Third	30 (75)	27 (67.5)
Fourth	25 (62.5)	20 (50)
Fifth	18 (45)	18 (45)
Sixth	17 (42.5)	17 (42.5)
Seventh	17 (42.5)	16 (40)
Eighth	16 (40)	16 (40)
Ninth	16 (40)	15 (37.5)
Tenth	14 (35)	14 (35)
Eleventh	14 (35)	14 (35)
Twelfth	14 (35)	13 (32.5)
Thirteenth	13 (32.5)	13 (32.5)
Fourteenth	13 (32.5)	13 (32.5)
Fifteenth	12 (30)	12 (30)
Sixteenth	12 (30)	12 (30)
Seventeenth	12 (30)	12 (30)
Eighteenth	11 (27.5)	10 (25)
nineteenth	7 (17.5)	7 (17.5)
Twentieth	7 (17.5)	7 (17.5)
Twenty-one	5 (12.5)	5 (12.5)
Twenty-two	5 (12.5)	5 (12.5)
Twenty-three	5 (12.5)	5 (12.5)
Twenty-four	2 (5)	2 (5)
Twenty-five	1 (2.5)	0

Table 2: Means and Standard Error for survival time

Mean		
Estimate	**SE**	**95% CI (lower bound-upper bound)**
392.416	3.885	384.802-400.029
420.000	6.682	406.904-433.096

SE: Standard error, CI: Confidence interval

worms was found to be 392 h with a confidence interval (CI) of 95% between 384.8 and 400.03. In addition, the median survival time for worms was calculated to be 420 h with a CI of 95% between 406.9 and 433.09 [Table 2]. Figures 2 and 3 show the cumulative survival and cumulative hazard of parasites according to time of cultivation, respectively.

Discussion

Cultivation is a crucial technique for the detection and fast diagnosis of the many clinically important parasites. Besides, cultivation also has enormous applications such as testing of vaccine efficacy, antigen production for getting serological techniques, detection of drug resistance, analyzing of possible therapeutic agents, and conducting epidemiological studies. *In vitro* cultivation of parasitic organisms has many benefits including enabling the realization of antiparasitic drug testing, parasite-host relationship, and transcriptomic studies or uptake of macromolecules by the parasite.[16-18] Cultivation of helminths has been often difficult because of their complex body structure and complicated life cycles. In addition, there are many conditions that need to be considered in the cultivation processes such as incubation time, nutrients, and temperature for various parasites. Even after decades of research in the field of human worms cultivation, only partial success has been achieved to culture parasitic helminths such as *Strongyloides* spp., hookworms, *Trichinella spiralis*, *Taenia* spp., and *Brugia malayi*.[9] However, up to now, no trematode species have been kept *in vitro* through the whole life cycle.[7] In the following, we will discuss some researches that have been done on various *in vitro* cultivation. Antiseptic techniques in bacteria-free media have prolonged the lifetime of the worms and in some species have resulted in the development from a larva to sexual maturity. Nevertheless, due to the complicacy of their metabolism and their inability to meet essential environmental conditions, in many researches, they have failed to complete their life cycles under synthetic conditions.[15] In 2004, Spiliotis *et al*. reported developing an *in vitro* system under axenic conditions for the long-term cultivation of *Echinococcus multilocularis* larvae.[14] The life cycle of *Strongyloides stercoralis* an important human pathogenic parasitic nematode is very sensitive to moisture, pH value, and nutrition conditions. While the Baermann technique is one of the most common methods for *S. stercoralis* cultivation, the agar plate culture technique has appeared as the more sensitive and reliable method.[21]

The other cultivation method is RPMI media 1640 which has a wide range of applications for mammalian cells including the culture of fresh human lymphocytes, fusion protocols, and growth of hybrid cells. For instance, it was used for maintaining and cultivating digenea.[11,22] Although adults of digenean *Microphallus turgidus* are successfully cultured in RPMI-1640, it was not a suitable culture for *Gynaecotyla adunca* because they were lived <4 days and produced few eggs.[11] Furthermore, encysted juveniles, young and adult *Fasciola hepatica*, are kept in RPMI 1640 supplemented with penicillin, streptomycin, serum, or RBCs.[23,24] In addition, adult *Schistosoma mansoni* is cultivated in DMEM, RPMI 1640, or Hanks' Balanced Salt Solution (HBSS) supplemented with Fetal Calf Serum (FCS), streptomycin, and penicillin.[25,26] On the other hand, *Schistosomes* can survive in a variety of supplementing tissue culture media from 3 to 120 days.[10] Culture media that are used for class trematodes are often supplemented with host tissue extract blood and serum.[22] West *et al*. were able to optimize conditions for *in vitro* culture of the microphallid digenean *Gynaecotyla adunca*. In this experiment, metacercariae of the parasite were cultured in different conditions to find those that resulted in the maximum worm longevity and egg production. When cultured in medium lacking serum, the worms lived longer in HBSS and Dulbecco's Modified Eagle medium/F-12 (DME/F-12) than in RPMI-1640 but produced the most eggs in DME/F-12. This research showed that worm longevity and egg production increased when worms were grown in DME/F-12 supplemented with

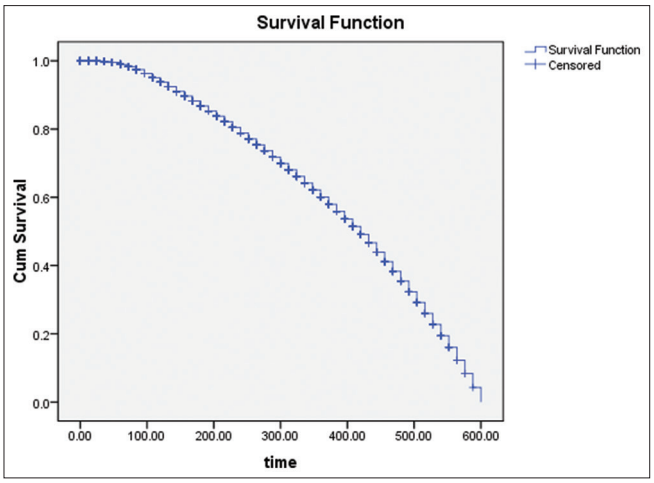

Figure 2: The cumulative survival of parasite according to time of maintenance in culture media

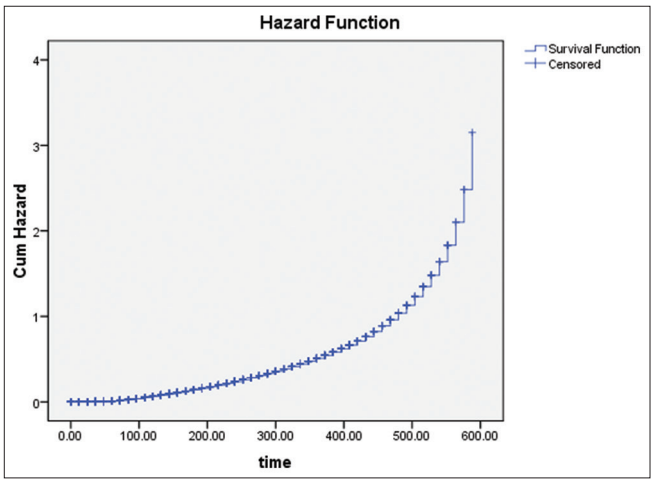

Figure 3: The cumulative hazard of parasite according to time of maintenance in culture media

20% chicken, horse, or newborn calf serum, but the greatest number of eggs was deposited in cultures containing horse or chicken serum. The optimal concentration of horse serum with respect to egg production ranged from 5% to 20%.[11]

Chaithong *et al*. were cultured newly excysted metacercariae of *Haplorchis taichui* in 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). This investigation showed that 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 BA without any change in result in a different concentration of blood in BA.[20]

The temperature of the culture media counts as one of the major factors for the cultivation of trematodes. Therefore, to perform successful cultivation, the temperature of the culture medium should be the same as the definitive host body temperature of the parasite. As a vivid instance, *Gymnophalloides seoi* (intestinal worm) was developed *in vitro* at 41°C better than 37°C.[27] *In vitro* cultivation of *Angiostrongylus cantonensis* with a complicated the cycle in the laboratory is one of the basic methods which could be applied to interpret the interaction among parasite and host. The time of living of larvae might be kept up for over 30 days by *in vitro* cultivation of L1 larvae in DMEM (DMEM basic, 20% FCS, 1% snail protein extract, 0.1% 5 mg/ml cholesterol-ethanol solution, mixed antibiotics: 100 units/mL of penicillin G potassium, 50 µg/mL of streptomycin sulfate, and 0.5 µg/mL of amphotericin B) and L3, L4, and L5 larvae in Waymouth's medium with 20% FCS and mixed antibiotics.[28] Native Toxocara excretory-secretory (ES) antigens have been used generally for diagnosing and seroepidemiological studies which are principally supported serological tests such as ELISA as well as western blotting. It is vital to obtain high quality of antigen-secreting Toxocara larvae from ES to cultivate the large amounts of *Toxocara cati*, second-stage larvae. Hence, the viable larvae were placed *in vitro* culture medium which consists of RPMI 1640, sodium bicarbonate, L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), penicillin/streptomycin, and 1% glucose.[29] In a laboratory study, cultivation of *Echinococcus granulosus* protoscoleces leading to adult worms was settled under aseptic conditions in a diphasic S.10E.H medium. For this purpose different forms of parasites including pre-segmentation stages and segmentation stages were evaluated in these medium. Finally adult worms contained four proglottids with a large and distinct genital pore were observed 50-55 days post cultivation.[30]

Conclusion

For the first time, in the current research, a culture medium for the *in vitro* study of *D. dendriticum* was developed by consecutively testing the suitability components of the medium. The results were shown that the RPMI 1640 supplemented with FBS and RBCs can be used as short-term maintenance for the *in vitro* culture of *D. dendriticum*. However, further studies are needed.

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Conflicts of interest

There are no conflicts of interest.

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