A new technique to avoid losing the strains of Echinococcus multilocularis during passaging

Tonay Inceboz, *MD, Phd,* ***Metin Korkmaz,*** *MD,* ***Ahmet Uner,*** *PhD.*

ABSTRACT

Objective: To examine the survival of *Echinococcus multilocularis (E. multilocularis)* and the formation of protoscoleces, under refrigerated conditions over a 43-day observation period.

Methods: We conducted this study in Ege University, School of Medicine, from May-December, 2004. We included 4 healthy females and 2 males, 8-12 week old *M. unguiculatus* species (approximately 60-70 gr) for the study, as they are known to be susceptible to *E. multilocularis.* In this experimental study, we aimed to define a technique to keep the strains during these passages.

Results: We found preserved viability and virulence of *E. multilocularis* stored in RPMI 1640 plus 10% fetal calf serum at $+4^0$C. We accomplished the infection of the *Meriones unguiculatus* after in-vivo passaging from that flask, which we kept at $+4^0$C, and this proves the viability of protoscoleces and membranous structures perfectly at the 43rd day.

Conclusion: We also found that *E. multilocularis* metacestodes and the formation of protoscoleces keep their viability up to 60 days and virulence up to 43 days under this condition. We recommend storing an extra flask during each passage as a precaution against losing strains.

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We keep *Echinococcus multilocularis* (*E. multilocularis*) metacestodes, which cause alveolar echinococcosis (AE), in laboratories for research purposes by serial passages in rodents.[1-4] To maintain the strains, we use different animals and methods. More commonly, researchers use mice or gerbils infected by intraperitoneal or intrahepatic injection of metacestode-infected tissue, which subsequently resulted in secondary AE.[5,6] However, previous studies demonstrated the potential feasibility of performing in vitro cultivation and proliferation of the metacestode stage of *E. multilocularis*.[7,8] The present study examined the survival of *E. multilocularis* and the formation of protoscoleces, under refrigerated conditions over a 43-day observation period. Microscopic examination of metacestodes and the formation of protoscoleces indicated that no visible development occurred under refrigerator conditions.

Methods. The study was carried out in Ege University, School of Medicine between May-December 2005. The study was approved by the local ethics. All animals were anesthetized with ether during the injection and the imaging process. All the experiments in this study were carried out in compliance with the relevant national laws relating to the conduct of animal experimentation. To protect the study personnel from AE infection, sterile gloves were used with protective masks for the face and the eyes to avoid any danger in case of splashing in the sterile conditions of the security chamber. The *E. multilocularis* metacestodes were

From the Department of Parasitology (Inceboz), Faculty of Medicine, Dokuz Eylul University, Inciralti and the Department of Parasitology (Korkmaz, Uner), School of Medicine, Ege University, Bornova, Izmir, *Turkey.*

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Address correspondence and reprint request to: Dr. Tonay Inceboz, Dokuz Eylul University, Department of Parasitology, Inciralti, 35340, Izmir, *Turkey*. Tel. +90 (232) 4124545. Fax. +90 (232) 2590541. E-mail: tonay.inceboz@deu.edu.tr/umits@egenet.com.tr

taken from stock infection in Mongolian gerbils (*Meriones unguiculatus* [*M. unguiculatus*]) and were grafted intraperitoneally into 3-month-old gerbils. Four healthy females and 2 males, 8-12 week old *M. unguiculatus* species (approximately 60-70 gr) were chosen for the study, as they are known to be susceptible to *E. multilocularis*. The exclusion criterion from the research was determined to be the presence of an infection or systemic reaction. Two months after infection, the gerbils were sacrificed by cervical dislocation, and metacestodes (which appeared as cysts) were collected, cleared from the surrounding tissue, and washed thoroughly in 0.9% sodium chloride.[9] Tissue blocks of *E. multilocularis* vesicles with a volume of 1 cm^3 were cut into pieces very carefully, with a sterile scalpel and then were placed in a Petri dish under the sterile conditions. In vitro cultivation at $+4^0$C of *E. multilocularis* metacestodes was carried out as described previously.[10] Two pieces of tissue were placed in 40 ml of culture medium (RPMI 1640 containing 10% fetal calf serum). The tissue blocks were kept in tightly closed culture flasks (75 cm^2) placed in an upright position in an incubator at $+4^0$C, with medium added to every controlled flask. We accomplished the infection of the *M. unguiculatus* after in vivo passaging from that flask, which was kept at $+4^0$C on 15, 30, 43 and 60 days. Approximately 1 cm^3 size of tissue infected with *E. multilocularis* for each animal are placed carefully in a Petri dish under sterile conditions and 2 cm^3 volume of physiological saline is added to the isolated material into the Petri dish to prepare inoculation material. One cc (approximately 1000 vesicles and protoscoleces) was inoculated into the peritoneal cavity of each anesthetized *M unguiculatus* by using a 16 G injector needle. The success of implantation after inoculation by injection is justified by the palpation of the masses identified in the abdomen within 50-60 days.

Results. After having protoscoleces of *E. multilocularis* cultivated in the first flask, all invaginated and evaginated forms were seen motile under the inverted microscope. Subsequently the flask was kept in in vitro conditions at $+4^0$C during the first 24 days. Then they were examined under the inverted microscope again, and there was no deformity found in protoscoleces and membranous structures. Even after thawing, it was observed that protoscoleces have started to move at the 40th minute, and 80% were motile at the 50-60th minute. Each time we have counted 100 protoscoleces in flask and examined their viability. During this observational period, we found that none of the protoscoleces were lost, instead all kept their viability at $+4^0$C in vitro. After day 20, protoscoleces started to lose their viability over the course of time. The *E. multilocularis* protoscoleces in culture were observed in flasks at $+4^0$C during 2 consecutive months and this observation showed that none of the protoscoleces lost viability in the first 3 days. **Table 1** illustrates the protoscoleces viability ratio. In days 1, 15, 30 and 43, we accomplished to infect *M. unguiculatus* after in vivo passaging from that flask, which was kept at $+4^0$C, and this proves the viability of protoscoleces and membranous structures perfectly. But, we have been unable to infect the *M. unguiculatus* after in vivo passaging from the flask in days 50 and 60. After the *M. unguiculatus* were infected, the vital functions of these experiment animals were examined from the first day. After the first month, the abdomen was palpated to check whether irregular masses had formed due to AE disease. Palpation of many irregular masses in the abdomen of the animal is proof of the development of AE. Approximately 2-3 months later, it was noted that the abdomen of the animal had grown, and its movements slowed down noticeably. An improvement in the general condition and a prolonging in their lives for approximately one week was observed in the experimentally infected gerbils when the infected material was taken out from the peritoneal cavity via operation despite being close to death. The *E. multilocularis* tumor masses were identified in the abdomen of dissected animals. An experienced person can reach the diagnosis by macroscopic observation. However, demonstration of protoscoleces formation vesicles with the microscopic examination makes the definitive diagnosis.

Table 1 - Microscopic assessment of viability of protoscoleces of *E. multilocularis* in flask during 60th days.

Time stored	Protoscolces viability ratio (%)
1/2 hour	(100)
3rd day	(100)
10th day	(90)
20th day	(84)
27th day	(80)
30th day	(60)
43rd day	(40)
50th day	(20)
60th day	(2)

Discussion. Viability and virulence of the pathogens are 2 important issues not only for infections, but also for experimental purposes. Ohnishi et al[11] investigated the viability and virulence of *E. multilocularis* at different temperatures. Casado et al[12] reported recovery of

metacestodes 270 days after mice inoculation of *E. granulosus* that they kept for 90 days in vitro.[12] In this study, we found that under in vitro conditions in RPMI 1640 with 10% FCS at +4^{0}C, protoscoleces and membranes of *E. multilocularis* lost all their viability at the sixtieth day. Interestingly, we injected a 1 mL sample of protoscoleces and membranes of *E. multilocularis* after 43 days in RPMI with 10% FCS intraperitoneally into the gerbil, and we could recover the strain on the sixtieth day after the injection. This proves the preservation of the virulence of the strain in vitro. Previous studies have proposed cryopreservation of *E. multilocularis* to prevent the loss of strains.[13,14] However, there still may be some loss of strains depending on the technical details. For instance, nitrogen tank, a sine-qua-none of cryopreservation, may be empty, and a detail as small as this may cause a loss of that strain. In addition, cryoprotectant substances may cause damage to the cells.

In the last few years, many studies performed the in vitro cultivation of *E. multilocularis*.[5,10,15,16] These investigations are very important because they do not only supply the maintenance of the strains but also give the opportunity to investigate the life cycle of the parasites and find appropriate drugs against such parasites. Comparing *E. multilocularis* in vitro cultivation and keeping at +4^{0}C for cultivation is disadvantageous because of the obligation of passaging every 3 days, as during the passages there is risk of infection of the person involved, the risk of contamination of in vitro culture with bacteria, and poor cost-effectiveness. However, by keeping at +4^{0}C, we minimize the risk of infection of the persons and the culture media, and this method is highly cost-effective.

There may also be the strain loses due to dying of the animals that we use during in vivo passaging of *E. multilocularis*.[2,6,9,17] In this perspective, we believe that the preservation at +4^{0}C is again advantageous. We hold the view that with this strain, we can obtain the antigens required for the serological diagnosis of AE from the indigenous strain.[18]

The main aim of all methods for preservation is the prevention of losing the strains. In addition, the ideal method should be easy, cost-effective and with minimal risk for personnel. Therefore, maintenance at +4^{0}C may be a good alternative for cryopreservation of *E. multilocularis.*

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