



Title	STUDIES ON ECHINOCOCCOSIS XX : PRELIMINARY OBSERVATIONS OF THE IN VIVO CULTIVATION OF LARVAL TISSUE OF ECHINOCOCCUS MULTILOCULARIS IN CULTURE-CHAMBER OF POROUS MEMBRANE
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Citation	Japanese Journal of Veterinary Research, 15(4), 165-169
Issue Date	1967-12
DOI	10.14943/jjvr.15.4.165
Doc URL	http://hdl.handle.net/2115/1888
Type	bulletin (article)
File Information	KJ00002369353.pdf



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STUDIES ON ECHINOCOCCOSIS XX
PRELIMINARY OBSERVATIONS OF THE IN VIVO CULTIVATION
OF LARVAL TISSUE OF *ECHINOCOCCUS MULTILOCULARIS*
IN CULTURE-CHAMBER OF POROUS MEMBRANE

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(Received for publication, October 11, 1967)

Various media and techniques have been used for the cultivation of the scolices of *Echinococcus multilocularis* or *E. granulosus* in vitro, and the vesicular development of scolices were observed by many investigators.^{1~4,7,8,11~14)} They, however, could not obtain any vesicle with a scolex, while LUKASHENKO (1964) obtained the vesicles having the scolices with hooklets by using the minced germinative tissue of vesicles without a scolex of *E. multilocularis*. SAKAMOTO et al. (1967), in cultivating the germinal cells of daughter cysts from cotton rats infested with *E. multilocularis*, observed a remarkable multiplication of the cells. The proliferation of the cells, however, declined generally with the passage of time and subculture, showing a tendency of dedifferentiation. The method used by LUKASHENKO (1964) did not level itself to the development of the vesicle with scolex in authors' attempt. The methods for the in vitro cultivation of echinococcal tissue should be improved in accordance with the essential physiological requirement of the tissue. It is also well known that the in vivo cultivation is carried out without trouble with the culture medium. Here, the authors attempted the cultivation of the larval echinococcal tissue in a chamber made of porous membrane which was inserted within the peritoneal cavity, and obtained a remarkable growth of tissue.

MATERIALS AND METHODS

The porous membrane, type HA of Millipore filter, is composed of pure and biologically inert cellulose ester, and is designed as $0.45 \pm 0.02 \mu$ in pore-size, $150 \pm 10 \mu$ in thickness and 9.6 mm in diameter. The porous membranes were pasted with MF cement (made by Millipore Filter Corp.) on both side of the silicone gum tube, 7 mm in length and inside diameter and 1.6 mm in wall thickness. An injection needle was inserted into the wall of culture-chamber. The culture-chambers were put in a Petri dish which was wrapped completely with a parchment-type autoclaving paper, and autoclaved for 15 minutes at 113°C.

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Culture materials used were collected aseptically from the liver of cotton rats experimentally infested with the eggs from *Echinococcus multilocularis* which were obtained from dogs in the Nemuro district, Hokkaido, Japan. Echinococcal tissue containing abundant scolices was treated with 0.2% trypsin solution, the tissue released was filtered by a stainless-steel mesh, and washed three times with HANKS' solution. For storage of the materials, the echinococcal tissue was incubated at 37°C for 3 to 7 days in cubic culture bottle (250 ml) containing the medium which consisted of equivalent HANKS' solution with 0.5% lactalbumin and 0.1% yeast extract and medium 199 added 20% calf serum. The echinococcal tissue, at the time of use, was removed from the culture bottle, washed well 3 to 5 times with HANKS' solution, and homogeneously mixed in medium 199. The suspension containing 300 to 500 scolices per ml, a small number of germinal cells and a few small pieces of brood capsule were introduced into the culture-chamber. Air in the culture-chamber was completely pushed out through the porous membrane by introduction of the suspension. When the injection needle was withdrawn from the wall of culture-chamber, the pore closed naturally, and was pasted with a small quantity of cement as a precaution. Prior to the time the chambers were set in the cotton rat, they were stored in the Petri dish containing medium 199. Three or 4 culture-chambers containing echinococcal tissue were inserted into abdominal cavity of an adult male cotton rat which was narkotized with ether gas and incised at a hypogastric median-line. The abdominal wall and skin were sutured using a continuous CUSHING's mattress and interrupted mattress stitch respectively. The cotton rats operated on did not show an abnormal condition for half a year. The morphological change of echinococcal tissue was examined by removing the culture-chambers from the cotton rats sacrificed in course of time. Observations were carried out extending over 4 days to 6 months using the routine and phase contrast microscopes. Supravital staining and hematoxyline-eosin staining were used for the sections and pressed specimens.

RESULTS

The culture-chambers set in the abdominal cavity of the cotton rat were enveloped with a membrane of connective tissue.

On the 3rd day, most scolices showed regressive changes and a small number of scolices revealed a vesicular development. The reticulate proliferation of germinal cells was observed among scolices.

On the 30th day, the echinococcal tissue settled on one of the porous membranes, and the inner space of the culture-chamber was completely filled up by the vesicles originating from the above tissue. The wall of the vesicle was thin, and the inner surface of the wall was lined with typical germinal cells. The accumulations of germinal cells considered identical with initial brood capsule formation were found on the inner surface.

On the 80th day, a number of mature brood capsules containing scolices developed fully, and numerous calcareous corpuscles were recognized. On the other hand, formation of many new brood capsules and daughter cysts were also found.

Six months after the insertion in vivo, the culture-chamber was full of many vesicles with a number of brood capsules containing many scolices. Considerable numbers of daughter cysts were recognized in the vesicles.

The authors could not find any multilocular vesiculation by exogenous budding such as hydatid cysts in the primary infection and the *in vitro* cultivation. The authors recognized hardly any difference in the development of vesicles among the culture-chambers from every one of the adult male cotton rats.

DISCUSSION

Up to the present, the *in vivo* cultivation of echinococcal tissue in a culture-chamber made of porous membrane has never been reported, as far as the authors know. In this experiment, the authors carried out the *in vivo* cultivation using the culture-chamber inserted in the abdominal cavity of the cotton rat. With exception of multilocular vesiculation by exogenous budding, the echinococcal tissue in the culture-chamber made of porous membrane revealed the development differentiating in the same manner as that of the infective lesion. There was little difference in the development of vesicles among the culture-chambers which were obtained from every one of the adult male cotton rats, as far as the present results reveal. On the other hand, though the cultivation of parasites *in vitro* will make clear the questions concerning their own physiology and biology, it will not produce the answers to many problems on the host-parasite relationships. Accordingly, it is considered that this *in vivo* culture technique may be applied to the various experiments such as investigations of host-parasite relationships, screening test of anthelmintics, research of immunology, etc.

Moreover, YAMASHITA *et al.* (1962) recognized that the subcuticular cells were simultaneously metamorphosed into the asteroid germinal cells *in vitro*. SAKAMOTO *et al.* (1967) could not observe the scolices and brood capsule differentiated from the germinal cells cultured *in vitro*, notwithstanding the cells revealed considerable proliferation. It may be considered that the cells are inclined to dedifferentiate *in vitro* while differentiating into various tissue *in vivo*. Accordingly, it can be conjectured that something that passed through the porous membrane, $0.45 \pm 0.02 \mu$ in pore-size, is concerned in the differentiation of the cells. In this experiment, however, it could not make clear whether scolices or germinal cells reticularly proliferated developed into the vesicles in the culture-chamber. The authors expect, in further observation, to ascertain what the matrix of the vesicles in the culture-chamber is.

In this experiment, the initial brood capsule formation and the completion of scolices within the capsule were found on the 30th day and the 80th day respectively. OHBAYASHI (1960) reported that he found the initial brood capsule formation on the 20th day and the completion of brood capsule with scolices 53~60 days after infection in the cotton rat. SADUN *et al.* (1957) found the immature scolices without hooklets in the cotton rat 6 weeks following inoculation

of *E. multilocularis*, and observed well developed scolices with hooklets on the second month after infection. So, the authors conclude that the echinococcal tissue in culture-chamber develops slowly more or less in comparison with that in cotton rats which were experimentally infested with eggs of multilocular echinococcus. The authors, in the future, will investigate the passage of hydatid tissue by this technique and the difference between development of hydatid tissue in the culture-chamber and that out of the chamber in intrahepatic and intra-peritoneal inoculations.

SUMMARY

Echinococcal tissue consisted essentially of 300 to 500 scolices, a few germinal cells and small pieces of brood capsule, was introduced into the culture-chamber made of porous membrane of $0.45 \pm 0.02 \mu$ in pore-size, composed of cellulose ester, and silicon gum tube, $3.5^2\pi \times 7\text{mm}^3$ in capacity. Three or 4 culture-chambers containing echinococcal tissue were inserted into abdominal cavity of a cotton rat, and the development of echinococcal tissue was observed morphologically in the course of time. The results obtained are as follows.

On the 3rd day, vesicles which were lined by typical germinal cells and showed initial brood capsule formation filled up the space of the culture-chamber. On the 80th day, many mature brood capsules with scolices fully developed, daughter cysts and numerous calcareous corpuscles were present in the vesicles. The culture-chamber was full of vesicles with a remarkable number of brood capsules containing many scolices after 6 months. No multilocular vesiculation by exogenous budding was recognized.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Dr. J. YAMASHITA, Professor of Department of Parasitology and Dr. M. OHBAYASHI, Assistant Professor of Department of Parasitology for their kind direction and review of this study.

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EXPLANATION OF PLATES

Figures 4~6 and 8 were photographed by the use of a phase contrast microscope. Figures 2, 10 and 11 are photographs of hematoxylin-eosin stained materials.

PLATE I

- Fig. 1 Culture-chambers with injection needle respectively in Petri dish
- Fig. 2 Reticularly proliferated germinal cells among regressive scolices, 3rd day, $\times 170$
- Fig. 3 Vesicles developed on porous membrane, 30th day, $\times 7$
- Fig. 4 Germinal cells lined on inner surface of vesicle, 30th day, $\times 630$
- Fig. 5 Accumulated germinal cells at initial brood capsule formation stage, 30th day, $\times 630$
- Fig. 6 Scolices and calcareous corpuscles in vesicles, 80th day, $\times 170$

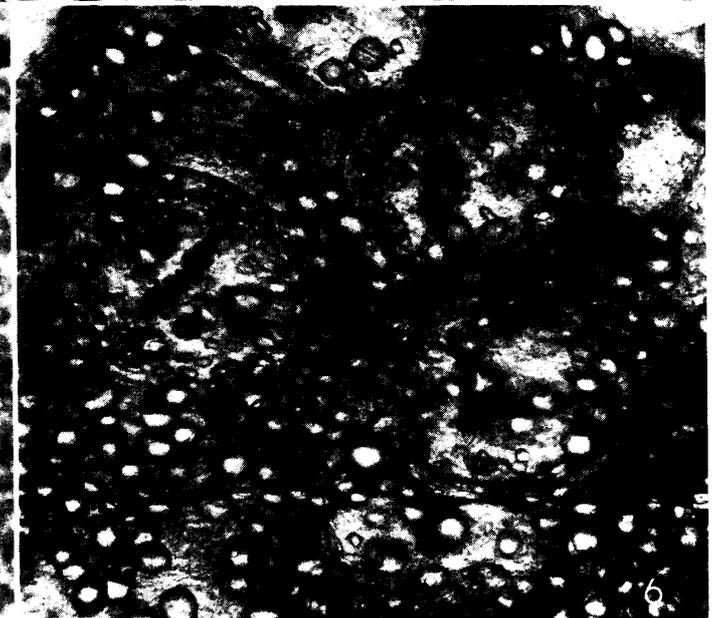
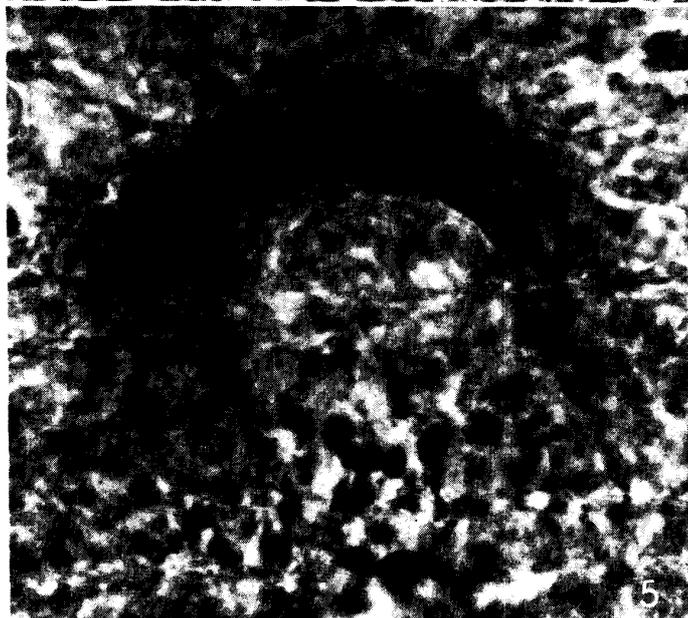
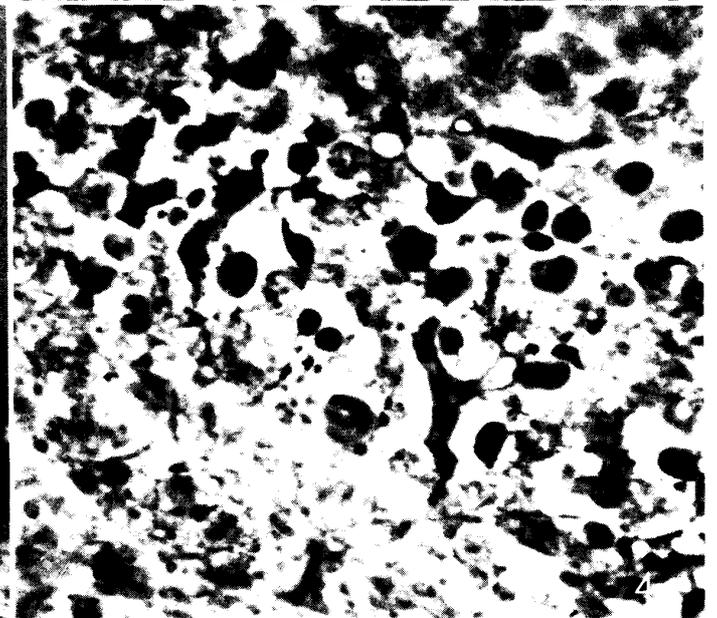
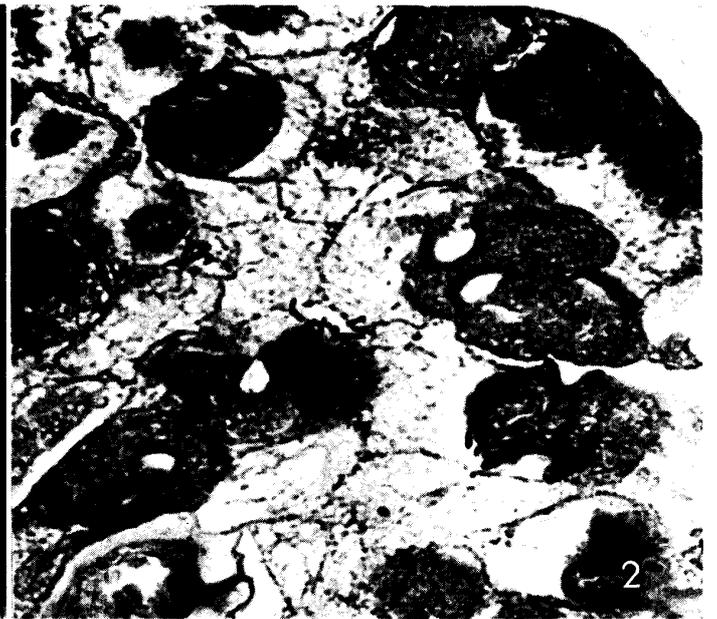
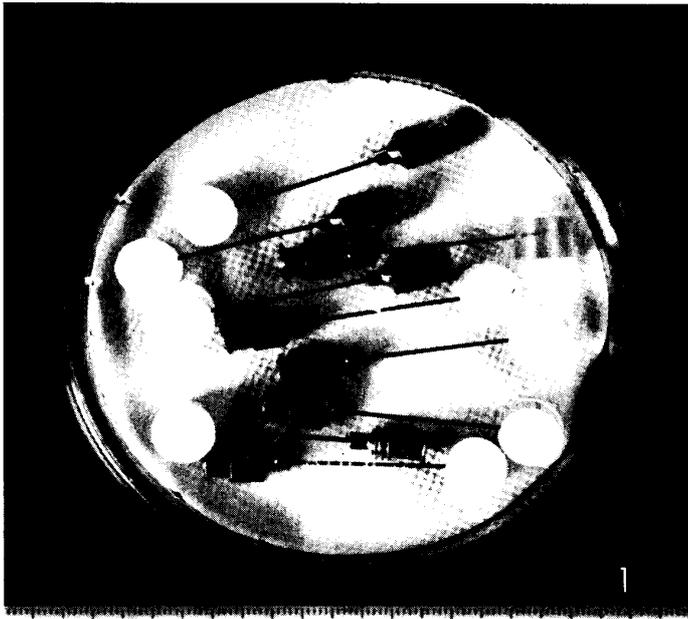


PLATE II

- Fig. 7 Daughter cyst obtained from vesicle, 80th day, $\times 68$
- Fig. 8 Formation of new brood capsule in vesicle, 80th day, $\times 360$
- Fig. 9 Vesicles containing many brood capsules (B) with scolices and daughter cysts (D), and scolices (S) released from ruptured vesicles, 6th month, $\times 5$
- Fig. 10 Vesicle containing brood capsule with scolices, 6th month, $\times 68$
- Fig. 11 Brood capsules with scolices in vesicles, 6th month, $\times 170$

