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Original Article

Biological Properties of Oncospheres of *Echinococcus* spp.

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ABSTRACT

Introduction: Taeniasis is known as a global human and animal parasite. Infestation by *Echinococcus* has significantly decreased in some countries as a result of modern research aiming to combat this type of tapeworm. To obtain protective biologics, biomass of inactivated material is required. The present study aimed to obtain the maximum number of oncospheres of the genus *Echinococcus* for the preparation of vaccines.

Materials and methods: *Echinococcus multilocularis* (*E. multilocularis*) eggs were placed in artificial intestinal juice. A beaker with juice and eggs was placed on a heated magnetic stirrer. To test the biological properties of the culture, *Echinococcus* eggs were used for infection of the laboratory mice. For infection, four groups of mice were used. *Echinococcus* eggs were activated in artificial intestinal juice on a magnetic stirrer in the first group. In the second group, cestode eggs were activated under thermostat conditions (38°C) in artificial gastric and, then, in intestinal juice. The third group was a positive control. The last group was a negative control. The selection of *E. multilocularis* metastases in laboratory mice was performed for 13 months. Cestodes parasitic cysts were evaluated in each infected mouse at the end of the experiment. The physiological status of *E. multilocularis* cysts in mice was assessed by a helminthological autopsy. The viability and activity of *E. multilocularis* protoscoleces were evaluated by motor activity. Mobility was recorded when heating the samples at 37°C for 10-15 minutes.

Results: In the first group, second, and positive control groups, 70%, 44.4%, and 33.3% of the mice were sensitive to the alveococcus causative agent, respectively. The larval cysts of the cestodes were identified in the liver and lungs of the mice in the first experimental group. In the second and third groups, the larval in the form of the alveococcus was identified only in the liver.

Conclusion: This method allows the investigation of the main biological indicators of cestode egg culture (viability, invasive activity). The novelty of the method is using only artificial intestinal juice without artificial gastric juice. The method can increase yield of activated oncospheres in a short period of time.

1. Introduction

Taeniasis is a global problem for humans and animals. The infestation of intermediate hosts by helminth larvae (*Echinococcus* and cysticerci) is much higher than what has been reported in medical and veterinary studies[1,2,3]. National prevention measures are needed to prevent this disease[4, 5]. Modern studies aimed to combat human and animal tapeworms, including *Echinococcus* tapeworm pathogen. However, they significantly reduced the level of invasion in most countries, such as Russia and the Commonwealth of Independent States, but it was less effective in Kazakhstan and Central Asian republics[6,7]. However, in most of these countries, infestation caused by *Echinococcus* tapeworms is hyperendemic to humans and animals.

Currently, the prevention of parasite infection is carried out by immunizing sensitive animals with oncospheres

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antigens (vaccines)[6,7,8,9]. Scientists use invasive tapeworm eggs to produce protective biologics. Reliable methods of determining the quality of biological material are required to assess the eggs or the tapeworm oncospheres. Previously, the viability and invasive capability of taeniid eggs were determined by appearance and staining with paints and setting a biological test [2,6,10]. The morphological structure of taeniid eggs does not provide reliable information about viable, dead, and dying embryos. A biological test on laboratory animals gives the result only after a few months but does not show the percentage of helminth quality eggs in the culture. Paint solutions (methylene blue, gencian violet, neutral red) color the eggs of dead tapeworms well and do not stain viable eggs and oncospheres[6,7,10]. In this method, the percentage of quality material is not clear. The most promising method of determining the viability and activation of tapeworm oncosphere is the cultivation method in natural or artificial digestive juices of hosts [6, 7, 11]. Activation of tapeworm eggs is determined by hatching the embryo from the eggshell and observing the physiological activity of oncospheres under the influence of temperature and/or enzyme factors.

The aim of the present study was to obtain information on the use of a fast one-step biotechnological method of activating cestode oncospheres of the genus *Echinococcus* to reduce the period of analysis of the presence of a full-fledged culture.

2. Material and Methods

2.1. Ethical approval

The present experimental research was conducted in compliance with health protection guidelines for experimental animals (Protocol of Invasion Diseases Section No. 1. 11/02/2019; European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. ETS No. 123. Strasbourg, 18/03/1986).

2.2. Delivery and preparation of material

This study aimed to show the artificially caused activity of eggs of higher cestodes. Table 1 shows the compositions of artificial intestinal juice (AIJ). *Echinococcus multilocularis (E. multilocularis)* eggs were placed in AIJ. A beaker with AIJ and eggs was placed on a heated magnetic stirrer (first experimental group). A magnetic stirrer modeled the digestion process in the helminth host. To compare the artificial activation process with other authors, a formulation of artificial gastric and intestinal juices with the addition of extra enzymes was used (second experimental group). The *E. multilocularis* eggs were gradually placed in digestive juices under thermostat conditions (38°C). Inactive *E. multilocularis* eggs isolated from helminth segments mechanically (positive control) were used as a control. In each group of experiments, 10 laboratory mice were used for helminth infection. To control the total mice stock of the experiment, a separate group (5 heads) was given saline orally (negative control). The selection of metacestodes *E. multilocularis* was carried out after the euthanasia of laboratory mice after 13 months of the experiment. The physiological state of helminth cysts and protoscoleces activity were studied.

The sexually mature helminths were examined to study the biological properties of *E. multilocularis.* Alveococcus tapeworms were obtained from the small intestine of the common five red foxes (*Vulpes vulpes*) in 2018-2020. Foxes were shot by hunters under one-time licenses (77.99.03.001. L.000067.08.13 of 28.08.2013) on the hunting farms territory in the Vladimir and Ryazan regions of the Central Region of Russia. More than 160,000 sexually mature tapeworms with mature segments were obtained by complete helminthological autopsy (K. I. Scriabin, 1928). The *E. multilocularis* invasive helminths were stored in distilled water in Petri dishes at a temperature of 6 ± 2 °C for 5-11 days[1]. Diagnostic tests and experimental data were carried out in the Federal State Budget Scientific Institution "Federal Scientific Centre VIEV" license laboratory (License number 77.99.03.001. L.000067.08.13 of 28.08.2013).

2.3. Preparation of artificial intestinal juice and biotechnological process.

The AIJ was prepared by adding 1.8 g of pancreatin, 1.2 g of edible soda, 24 ml of freshly obtained (thawed, concentrated) ruminant bile, and 20 ml of EDTA trypsin to 100 ml of distilled water (Table 1)[7,8,14]. The resulting mixture was poured into a magnetic stirrer, turned on the magnet rotation mode to 500 rpm, and held for 5 minutes. Then, AIJ was filtered through filter paper for 1 hour. After this procedure, the prepared solution became clear with a greenish tint. In the next step, 50 ml of filtered AIJ was pipetted into a 150 ml beaker. A 2.5 cm magnet was placed on the bottom of the glass by an anatomical tweezer. The examined *Echinococcus* tapeworms or their segments with eggs were put in the glass with a Pasteur pipette.

2.4. Preparation of magnetic stirrer for operation and artificial peptolysis process

In this study, magnetic stirrer IKA model RT-10 (China)

Table 1. Composition of artificial intestinal (duodenal) juice for activation of *Echinococcus* tapeworm oncosphere (Russia, red fox)

In order	Reagent	Quantity	Country of manufacture
1	Pancreatin with proteolytic activity 200 FIP units, powder	1.8 g	China
2	Soda food	1.2 g	Russia
3	Trypsin-EDTA, 0.25% solution, for cell cultures, sterile, Sigma-Aldrich	20 ml	USA, Germany
4	Bile of cattle, concentrated, sterile	24 ml	Russia, Volgograd
5	Distilled water	53 ml	-

FIP: Fédération International Pharmaceutique

with heating was used. The device was put into operation. A beaker of intestinal juice, invasion material, and a magnet was placed on the surface of the stirrer at 40±1°C and held at 50 rpm for 15 minutes. At the same time, the intestinal juice temperature inside the cup was monitored with a thermohygrometer with a remote temperature and humidity sensor (digital thermometer-hygrometer KTJ TA138, China). The temperature inside intestinal juice was 38±1°C. After exposure, a sample of the invasive material was pipetted and placed on a glass object, microscopic at magnifications x 10, and x 40.

2.5. Analysis

Embryos that were not released from the shells of eggs or those released but at the same time had gigantic sizes were considered dead. Released larvae with characteristic oval embryo morphology and three pairs of germinal hooks located parallel or at an angle of 45° were considered viable. Oncospheres exhibiting motor activity were considered activated.

The analysis result was recorded using Formula 1[6,9]:

$$P = \frac{V\,(A)}{T} \text{ x } 100\% \text{ [1]}$$

Where, P is the biological property of the oncospheres of cestodes, V denotes the number of viable oncospheres, A signifies the number of activated oncospheres, and T refers to the total amount of invasive material taken from the sample (eggs).

The mean viability and/or activation index was determined. The number of oncospheres that emerged from eggs was considered viable. The number of oncospheres with motor activity outside the egg was considered activated. The viability and activity of oncospheres were calculated using Formula 1.

2.6. Bioassay

Laboratory male mice were purchased from a specialized nursery in the Kroll-Info, Moscow, Russia. The mice were 3 months with a mean weight of 19 g. Linear mice of the inbred line BALB/CJLac (35 heads) included in this work were used for the bioassay. Mice were infected with *E. multilocularis* eggs (oncospheres). In the first experiment, eggs in AIJ were activated. In the second experiment, the eggs were activated first in gastric and then in intestinal juice, adding an additional number of enzymes. Upon activation, stationary or mobile oncospheres of *E. multilocularis* were obtained. In the first control, *E. multilocularis* eggs were not activated. The dose of the oncosphere of helminth *E. multilocularis* for laboratory rodents was calculated in the Migacheva-Kotelnikov counting chamber (1976). In the experimental groups, the infection dose was 550 tapeworm oncospheres per laboratory mouse (Table 2). The first experimental group of mice was given oncospheres activated according to the proposed one-step method. Activation of the *E. multilocularis* oncospheres in the second experimental group of mice was carried out according to known two-stage methods [7, 8]. The authors of the presented methods activated *E. multilocularis* eggs by replacing artificial gastric juice with intestinal juice. They use additional components (lipase) to produce intestinal juice. In the test, two experimental groups of mice were affected by *E. multilocularis* infection (in the first experiment, eggs activated in AIJ on a magnetic stirrer were used, in the second experiment, eggs activated in artificial gastric and intestinal juice without a magnetic stirrer). The first control group of mice (control 1) was infected with the same dose (550 specimens) of *E. multilocularis* by unactivated eggs. Helminth eggs were prepared by breaking the entomological needle of mature cestodes segments on a glass object [7, 8]. The second control group of mice was administered saline orally. Laboratory mice were infected with *E. multilocularis* following safety guidelines for handling invasive material[12]. Finally, after 13 months of infection, laboratory rodents were euthanized.

2.7. *Evaluation of the physiological state of E. multilocularis cysts*

Cestodes parasitic cysts were evaluated in each infected mouse at the end of the experiment. The physiological status of *E. multilocularis* cysts in mice was assessed by helminthological autopsy. The cyst was considered fertile when clear fluid, protoscolexes, hydatidosis sand, acephalocysts, and germinal membranes were found inside the cyst. The cyst was considered sterile if fluid and embryonic membranes were found in the cyst. The cyst was considered calcified when a cloudy liquid, a yellow curd content, was found in the cyst.

The viability and activity of *E. multilocularis* protoscoleces were evaluated by motor activity. Mobility was recorded when heating the samples at 37 °C for 10-15 minutes[6, 7, 14].

Table 2. Laboratory mice infestation plan (Russia, September 2020)

Groups	Quantity	Dose of infection	Viable (%)	Activated (%)
Expe-rience No.1	10	550 oncosp-	95	65
Expe-rience No.2	10	heres	35	0
Control No. 1	10	550 eggs	-	-
Control No. 2	5	-	-	-

3. Results

On days 3 and 21, one mouse died in groups 2 and 3, respectively. No lesions of internal organs with *Echinococcus* and helminth cysts were found during a postmortem sectional autopsy of mice carcasses.

In the first group, second, and positive control groups, 70%, 44.4%, and 33.3% of the mice were sensitive to the alveococcal pathogen (Table 3). In the first experimental group of mice, cestodes were recorded in the liver and lungs of mice. In the second experimental group and positive

control groups, the larval form of the chain was recorded only in the liver.

Table 3. Results of infection with *E. multilocularis* oncospheres and eggs of laboratory BALB/CJLac mice

Groups	Number of mice	Sensitivity (%)	Physiological status of *E. multilocularis* cysts		
			with calcium salts	sterile	fertile
First	10	70	-	-	7/10*
Second	10	44,4	1/9*	1/9*	2/9*
Positive control	10	33,3	1/9*	1/9*	1/9*
Negative control	5	0		0/5*	

* number of infected animals/total number of animals

4. Discussion

Of 10 mice in the first experimental group, fertile cysts were recorded in 7 animals (70%). In the second experimental group, fertile parasite cysts were registered in 2 mice, sterile cysts of the parasite in the first mouse, and cysts with the presence of calcium salts in the second mouse. The physiological status of alveococcal cysts in animal control 1 was different. No parasite cysts were found in the negative control. The experimental data obtained are consistent with the results of other authors[7, 8, 14].

Mature white chain larvae (fertile cysts) had fully developed and viable protoscoleces of the pathogen, acephalocysts, and hydatidosis sand (motor activity was recorded when the content of cysts was heated at 37°C). The size of the larvocysts ranged from 0.5 to 3.5 cm in diameter. Sterile cysts contained a good blood supply, had a pink color, and contained parasite acephalocysts. Sterile cysts (cysts of pink color) looked the same as fertile cysts and contained only cysts (diameter of 0.5-1.0 cm). In the extracted hydatids (yellowish-white color), there was a connective tissue shell with a small amount of cloudy liquid and calcium salts (cysts diameter of 0.1-0.5 cm).

Analyzing previous studies on the activation of the oncosphere of chains [7, 8, 11, 13, 14], the proposed formulation has a number of advantages (Table 1). To activate the invasive form of cestodes, the stage of changing enzyme solutions (digestive juices) can be removed in the volume of infection material. This technological method eliminates the process of preparation of cestode segments, which makes it possible to speed up research work, reduces losses of the invasive form of the pathogen, and ensures the safety of personnel from infection with invasive material. Mature segments, immature cestodes, and membranes of the pathogen eggs lyse quickly under the presented conditions since the circular movements of the instrument anchor (stirrers) create an analogue of the peristalsis of the intestine of the helminth host.

5. Conclusion

This method can investigate the main biological indicators of cestode egg culture (viability, invasive activity). The proposed method can increase the percentage yield of purified activated oncospheres from the shells in a short period.

With the help of the proposed method, it is possible to obtain biomass of activated chain oncospheres from nature, infect laboratory mice and obtain the volume of larvocysts for genetic typing of the pathogen and development of diagnostic antigen of *Echinococcus* chains in laboratory conditions.

This method allows you to obtain a full-fledged antigens material for synthesizing vaccines and diagnostics against flat helminths. Preparation in a large amount in a short time of biological material can be used in the industrial production of immunopreparations.

Declarations

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors conceived and designed the study. Andreyanov O.N. delivered, received biological material, and conducted an analysis of the research results. Postevoy A.N. conducted experiments on a laboratory model. All authors read and approved the analyzed data and the final revised article.

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