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A Simple Technique for Purifying Larval Nematodes

TOSHIO SUZUKI
Department of Medical Zoology, Niigata University School of Medicine, Niigata, Japan

S. Y. LEE, K. H. LIU and P. T. TSENG
Taiwan Provincial Malaria Research Institute, Taipei, Republic of China

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In experiments using larval nematodes it is desirable to isolate the larvae in pure form. This is especially true in physiological or biochemical studies as contamination by a small amount of impurities may exert an important influence upon the results. We have experienced difficulty in purifying first- and third-stage larvae of *Angiostrongylus cantonensis* for use in immunological studies. Because of these difficulties an attempt was made to discover a better method for the purification and concentration of nematode larvae.

Recently, Nakabayashi and Motomura (1968) reported a technique for separating cysts of *Toxoplasma gondii* from infected mouse brains by multi-layer centrifugation with Gum arabic solution. Subsequently, Takeuchi (1971) reported a purifictation technique of *Toxoplasma* trophozoites from the peritoneal exudate of infected mice applying centrifugation with Tris-sucrose-EDTA solution. Even though the materials utilized in these experiments were different the principles of purification were similar and the organisms were separated from the host components by the differece of specific gravity between them.

We wish to report here the successful purification of various nematode larvae from their host components using the same principles and the infectivity of these larvae subsequent to purification.

Materials and Methods

Larval nematodes

Third-stage *A. cantonensis* larvae were collected from naturally infected land snails, *Achatina fulica*, from Southern Taiwan. Snails were removed from their shells, coarsely minced with a large kitchen-knife and digested in a solution containing 1 % pepsin and 7 % HCl at 37°C for one and a half hours on a magnetic stirrer. The suspension was then strained through a fine metal mesh and the filtrate contrifuged at 2,000 rpm for 5 minutes. The sediment was then resuspended in 19 volumes of physiologic saline.

First-stage larvae of *A. cantonensis* were obtained from fresh fecal pellets of experimentally infected Long Evans rats. Fecal pellets were suspended in a large volume of dechlorinated water, strained through a fine metal mesh, and the filtrate centrifuged at 2,000 rpm for 5 minutes. The sediment was then resuspended in 19 volumes of dechlorinated water.

Hookworm larvae were obtained by the cultivation of pooled human stools obtained from infected persons. Fresh stools were suspended in a large volume of dechlorinated water and strained through a fine metal mesh. The filtrate was allowed to stand for 30 minutes, after which the supernatant was decanted. This procedure was repeated 5 times. The sediment was then incubated at 30°C for 10 days and the larvae were collected using Bearmann's apparatus.

Fertilized eggs of *Ascaris suum* were obtained from uteri of adult female worms collected in a local slaughter-house. The eggs were cultivated in an incubator (30°C) for 3 weeks, after which a large number of embryonated eggs were given via intra-esophageal tube to an adult albino rabbit. Three days after infection the rabbit was killed, the lungs removed, and larvae collected from the minced tissue using Bearmann's apparatus.

Soil nematodes were obtained from a clod of moist soil from a vegitable garden in the surburbs of Taipei City. The larvae were collected in the same manner as applied to the hookworm larvae.

Preparation of Gum arabic solution

To about 40 g of Gum arabic powder 100 ml of distilled water was slowly added with continual agitation by a glass rod. The solution was then boiled until thoroughly dissolved. After centrifugation of the solution at 3,000 rpm for 20 minutes pH of the supernatant was adjusted to 7.4 with saturated NaOH solution. The solution was then dialyzed against a large volume of phosphate buffered saline pH 7.4 (PBS) for 24 hours. Solutions with specific gravity of 1.100 and 1.070 (at 15°C) were made using PBS.

Purification of larvae from impurities

The heavier solution (s.p.: 1.100) was poured into a centrifgal tube and the lighter solution (s.p.: 1.070) was layered on it, finally the sample solution was laid on top. The solutions were poured slowly along the inner wall of tube with a pipette so that a clear demarcation between layers was seen. After centrifugation of the layered solution at 10,000 G for 15 minutes the larvae were concentrated in a faint zone between the bottom and middle layers. The larvae were carefully collected with Pasteur's pipette. A large volume of PBS was added to the collected sample and allowed to stand for 30 minutes, after which the supernatant was carefully pipetted off. This procedure was repeated 5 times.

Infectivity tests of purified A. cantonensis larvae

In order to study the infectivity of purified first-stage larvae, terrestrial snails, *Bradybeana similaris*, collected in Nangkan district, north-eastern Taipei, were used. This snail has been previously recognized to be susceptible to first-stage *A. cantonensis* larvae (unpublished data). Purified larvae were applied to leaves of lettuce which were then fed to 100 snails. On the 35th day after exposure 25 snails were crushed and emersing larvae were counted. As a control, other group of 100 snails were given lettuce leaves smeared directly feces containing larvae of *A. cantonensis*.

Infectivity of purified third-stage *A. cantonensis* larvae was determined in infecting Long Evans rats. One hundred purified larvae were fed to each of 10 rats by means of an esophageal tube. The rats were killed 45 days later and the lungs were carefully examined for worms. As a control, the same number of rats were infected similarly with initial samples.

Results and Discussion

Determination of specific gravity

Several trials were necessary in order to determine the optimal values in specific gravity of Gum arabic solutions to be used.

From initial experiment with first-stage *A. cantonensis* larvae it was determined that the combination of layered solutions with specific gravity of 1.090 and 1.070 concentrated the larvae in a sharp zone between these two layers. However, it was found that the recovery rates of third-stage *A. cantonensis* larvae and of hookworm larvae were low using these concentrations. Elevation of the specific gravity of the bottom layer to 1.100 improved the recovery rates for them. These layered solutions with specific gravity of 1.100 and 1.070 were used for subsequent tests.

Although tests were also carried out using sucrose solution of Gum arabic solution, satisfactory results were not obtained. The recovery rate of larvae was considerably

lower than with Gum arabic solution as the larvae did not form a clear zone between the two solutions but were distributed widely throughout the bottom layer. However, if the specific gravity of bottom layer was elevated to 1.130 the recovery rate increased but an amount of admixed debris also increased. As discussed by Nakabayashi and Motomura, the viscosity of the solution plays probably a significant role in the course of the separation similarly as the specific gravity does.

Concentration of sample

As another factor having an effect upon the recovery rate of larvae is the concentration of the sample suspension. When a dense suspension containing larvae was used, the larvae were enveloped with debris and precipitated together with them, and much of these admixtures also remained with the larvae. To collect pure larvae the quantity of charging material should be within approximately a 5% concentration and 2 cm in height.

Infectivity of purified A. cantonensis larvae

The prevalence and the intensity of infection in snails exposed experimentally to the first-stage larvae is presented in Table 1. There is a considerable difference in the number of larvae recovered between the snails exposed to purified larvae and those exposed to rat feces. As there is no means to ascertain the numerical relationship between the first-stage larvae taken by snails and the third-stage larvae detected in it, we are unable to quantitatively evaluate the infection rate. However, it appears that this technique does not greatly reduce the infectivity of firststage larvae.

There was no significant difference in the number of worms recovered between rats infected intra-esophageally with 100 purified third-atage larvae and rats given the same number of larvae from initial sample (Table 2). It therefore appears that larvae can be collected by this technique without losing their infectivity. Both the saline and the Gum arabic solutions were adjusted to isotonicity in this experiment. No studies, however, were undertaken to determine if this in any way affected the vitality of the larvae.

Summary

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Table 1 Prevalence and intensity of infection with third-stage *A. cantonensis* larvae in snails exposed to purified first-stage larvae and feces of infected rats

Snails exposed to	No. examined	No. positive	No. larvae per positive snail	
			Mean	Range
Purified larvae	25	25	18	2- 46
Feces of infected rat	25	25	44	2-120

Table 2 Recovery of adult worms of *A. cantonensis* from rats infected with purified third-stage larvae and larvae from initial sample

Rats infected with	No. examined	No. positive	No. worms recovered	
			Mean	Range
Purified larvae	10	10	47	31-62
Larvae from initial sample	9*	9	43	28-70

* One rat died 10 days after infection

nematodes utilizing 5 kinds of larvae is described. Larvae successfully purified included: first- and third-stage larvae of *Angiostrongylus cantonensis*, infective larvae of hookworm, lung-migrating larvae of *Ascaris suum* and soil nematodes. Larvae were concentrated in the sharp zone between two layers of Gum arabic solutions with specific gravity of 1.100 and 1.070 after centrifugation at 10,000 G for 15 minutes. Infectivity of the the purified first- and third-stage larvae of *A. cantonensis* was compared with those from initial samples. It appears that this technique does not cause apparent damage to the larvae as there was no significant differences in the infectivity between the two groups tested.

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References

1) Chiu, J. K. (1964): Snail host of *Angiostrongylus cantonensis* in Taipei, Taiwan. Bull. Inst. Zool., Academica sinica, 3, 55-63.
2) Kimura, E., Emoto, I., Awane, H. and Kinoshita, Y. (1960): Studies of functions of leucocytes separated from whole blood. 1. A method for separating live leucocytes from whole blood by means of multilayer centrifugation. Osaka City Med. J., 6, 49-58.
3) Nakabayashi, T. and Motomura, I. (1968): A method for separating cysts of *Toxoplasma gondii* from the infected mouse brains by multi-layer centrifugation with Gum arabic solution. Tropical Medicine (Nagasaki), 10, 72-80.
4) Takeuchi, T. (1971): Purification of *Toxoplasma* trophozoites from the peritoneal exudate of infected mice. Jap. J. Parasit., 20, 205-214.
5) Wallace, G. D. and Rosen, L. (1969): Experimental infection of Pacific island mollusks with *Angiostrongylus cantonensis*. Am. J. Trop. Med. & Hyg., 18, 13-19.

幼線虫の簡単な純粋収集法

鈴木　俊夫
新潟大学医学部医動物学教室
李松玉・劉国輝・曾柏村
台湾省マラリア研究所

幼線虫を用いて実験を行う際，幼虫はできるだけ純粋に，しかもそれらの生活力を侵すことなしに収集することが望まれる．

こうした必要性から以下の実験がなされた．比重の異なる二種のアラビアゴムの重層溶液に幼虫を含む試料懸濁液を重層して遠心することによつて幼虫のみを極めて純粋に収集できることが知られた．この実験に使用された材料は，広東住血線虫第一期ならびに第三期幼虫，鉤虫感染幼虫，ブタ回虫第三期幼虫，土壌線虫幼虫であるが，これらの幼虫はいずれも比重1.100および1.070のアラビアゴム液の境界に集まるとが知られた．

また，収集した広東住血線虫の両期幻虫を用いてそれぞれの宿主に対する感染性を調べたところでは，幼虫はこのような収集処理によつて著しい障害を受けることはないと考えられた．

Figure 1 First-stage *A. cantonensis* larvae.
Figure 2 Third-stage *A. cantonensis* larvae.
Figure 3 Infective larvae of hookworm.
(All figures enlarged 100×)