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# SURVIVAL OF FIRST-STAGE LARVAE OF *ANGIOSTRONGYLUS CANTONENSIS* (NEMATODA: METASTRONGYLIDAE) FROZEN BY A THREE-STEP PROCEDURE

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**David Chao, Kao-Cheng Chang and Iris T. C. Huang** (1988) Survival of first-stage larvae of *Angiostrongylus cantonensis* frozen by a three-step procedure. *Bull. Inst. Zool., Academia Sinica* **27**(2): 119-125. Attempts have been made to raise the survival rate of first-stage larvae of *Angiostrongylus cantonensis* at freezing temperatures. Parasites were obtained from feces of experimentally infected rats. The toxicity of two cryoprotectants was determined. A higher toxicity was observed when the concentration of cryoprotectant additive was higher than 30%. The muximum nontoxic concentration for DMSO in NCTC 109 medium was 10%. Different animal sera were used to test the survival of larvae after a −70°C storage. Heat-inactivated rabbit serum showed the highest protective potential. Therefore, a freezing medium containing 10% DMSO and 20% heat-inactivated rabbit serum in NCTC 109 was used to test the survival rate of the parasite after various freezing procedures. A higher survival rate was obtained when parasites were treated with freezing medium at 0°C for 5 min, held at −15°C intermediate temperature for 15-20 min before cooled to −17°C rapidly. A further assessment of viability of the thawed larvae was carried out by snail infections. Among 30% motile larvae recovered from thawing, 20% developed into third-stage larvae in planorbid snails.

**Key words:** *Angiostrongylus cantonensis*, Cryoprotectant, Survival rate, Cryopreservation, First-stage larvae.

The employ of freezing techniques for preservation of living parasites was first started with monkey malaria parasites, *Plasmodium inui* and *Plasmodium knowlesi* (Coggeshall, 1939). Extensive methodological studies on cryopreservation of various protozoa have been carried out for more than four decades (Diamond, 1964), while progress in freeze-preservation of parasitic helminths is still limited.

*Angistrongylus cantonensis*, the primary causative agent of human eosinophilic meningoencephalitis, is widely distributed in the regions of the South Pacific and Southeast Asia. Proven cases of human infection in southern and eastern Taiwan are increasing recently (Yii *et al.*, 1975; Chen, 1979; Chiu *et al.*, 1981; Huang *et al.*, 1984, 1986). Reliable cryopreservation techniques can facilitate the transport and storage of living *A. cantonensis* for various research applications but the development of this technique involves many aspects such as protective agents and their use, storage temperatures and their durations, and methods of freezing and thawing.

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Uga *et al.* (1983) have reported on the survival of 32-cell stage eggs of *A. cantonensis* from the thawing of frozen samples. However. they judged the viability of thawed eggs by somewhat growth, regardless of extent, within 48 hr in culture, but not by their ability of further development in snails. In addition, obvious difficulties are confronted by investigators in obtaining these 32-cell stage eggs. The investigators have no choice but to use other stages in the *A. cantonensis* life cycle to avoid the tedious procedures in dissection and *in vitro* cultivation. The present study describes the successful cryopreservation of first-stage larvae obtained directly from rodent fecest and the assessment of their viability by motility and by further development to infective third-strage larvae in the snail hosts.

## MATERIALS AND METHODS

### Parasites

The Taiwan strain of *Angiostrongylus cantonensis* (Chen) used in this study was originally isolated from the giant African snail, *Achatina fulica* (Bowdich) collected from Nei-hu, Taipei. It was maintained in this laboratory by cycling through the planorbid snail, *Biomphalaria glabrata*, and Sprague-Dawley rats (Animal Center, National Yang-Ming Medical College). Rats were infected with 40 third-stage larvae by stomach intubation, First-stage larvae were collected from feces of rats 65 to 85 days after infection, washed twice with normal saline, and employed in this study.

### Toxicity tests

First-stage larvae of *A. cantonensis* were incubated with various concentrations of cryoprotectants, dimethyl sulfoxide (DMSO, Sigma Chemical Company, St. Louis, Missouri) or glycerol, to determine the toxicity of these two cryoprotective agents. The parasites were suspended in 1 ml of Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, New York) or NCTC 109 (Microbiological Associates Bioproducts, Walkersville, Maryland) containing 5, 10, 15, 20, 25, or 30% (v/v) either DMSO or glycerol. The incubation was continued at room temperature for 30 min with frequent agitation. Ten ml normal saline was added rapidly at the end of incubation period to dilute the cryoprotective agent and the parasite suspension was centrifuged at 900 g for 7 min to remove DMSO or glycerol. The motility of the larvae was observed under a Nikon dissecting microscope.

### Freezing

The first-stage larvae of *A. cantonensis* were suspended in NCTC 109 medium concontaining 10% DMSO and 20% heat-inactivated rabbit serum and kept on ice bath for 5 min to equilibrate during which time the larval suspension was gently mixed at regular intervals. They were then placed in 1.2 ml Nunc conical bottom crew cap cryotubes at 0°C. The tubes were incubated at −15°C intermediate temperature in a Forma freezer for various intervals and devided into two groups Tubes in one group were thawed to determine maximum holdng time at −15°C for the survival of larvae after thawing. Tubes in the other group were cooled to −70°C in a Kelvinator ultracold cabinet. Samples cooled directly to −70°C without incubating at an intermediate temperature were used as controls. The optimum duration at the intermediate temperature for the viability of frozen and thawed larvae was determined after preserved at −70°C for at least 4 days.

### Thawing and dilution

After storage at −70°C the contents in the cryotubes were thawed rapidly by immersing the tubes in water bath at 37°C and simultaneously diluted in 37°C saline. The larvae were washed twice by centrifugation in saline to remove the cryoprotectant and assessed for their viability.

**The effect of different sera on cryopreservation**

In order to develop a technique for optimal recovering of viable larvae from forzen storages, the effect of sera from different mammalian species on the survival of cryopreserved parasites were compared. First-stage larvae were forzen in 1 ml NCTC 109 medium containing 10% DMSO and 20% heat-inactivated sera from dog, fetal calf, goat, monkey, and rabbit. Heat-inactivation of these animal sera were carried out at 56°C for 30 min. Samples frozen without serum and samples frozen with normal rabbit serum, not heat-inactivated, were used as controls. The survival of thawed parasites was examined after storage at −70°C for at least four days.

**Viability assessment**

The level of parasite survival was assessed in two ways. In motiliy assessment, thawed larvae were scored microscopically as: Grade 1, lively- essentially normal in appearance, locomotion, and gross morphology; Grade 2, abnormal- misshapen, motile, but the movement or shape changed; or Grade 3, dead-motionless. Only those scored in group 1 were counted as normally motile larvae and the number was expressed in percentage. For the foregoing assessment, the scoring time was 30 min after thawing. In the infectivity assessment, laboratory-hatched *B. glabrata* were infected with larvae recovered from cryopreservation according to the three-step freezing schedule. Each snail was exposed to forty thawed larvae pooled from samples held at −15°C for 15 and 20 min before cooled to −70°C. Infected snails were maintained at 27°C. The percentage of larvae developed into third-stage larvae in this planorbid snail was examined 40 days after exposure. Unfrozen larvae were used as controls.

## RESULTS

The toxicity of the two cryoprotectant additives, glycerol and DMSO, to the first-stage larvae of *A. cantonensis* in NCTC 109 medium or DMEM was shown in Fig. 1 or Fig. 2, respectively. In NCTC 109 medium, the best protective effect occurred in both of the two cryoprotectants with a concentration less than 10% additives. The highest toxicity occurred when glycerol concentration was higher than 30% in NCTC 109 medium (Fig. 1). DMSO has better cryoprotective effect in DMEM with a concentration less than 20% (Fig. 2). Therefore, from these reults DMSO at the final concentration of 10%

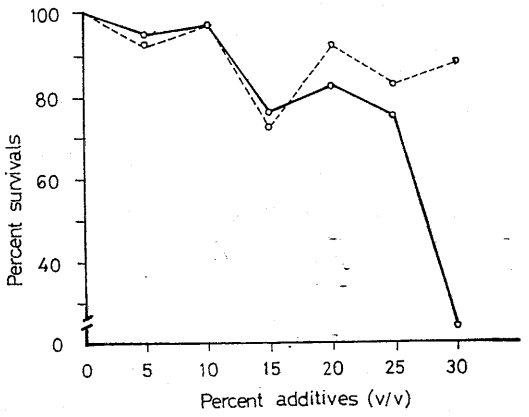


Fig 1. Toxicity of cryoprotective additives, glycerol (solid line) and DMSO (dashed line), to *Angiostrongylus cantonensis* larvae in NCTC 109 medium.

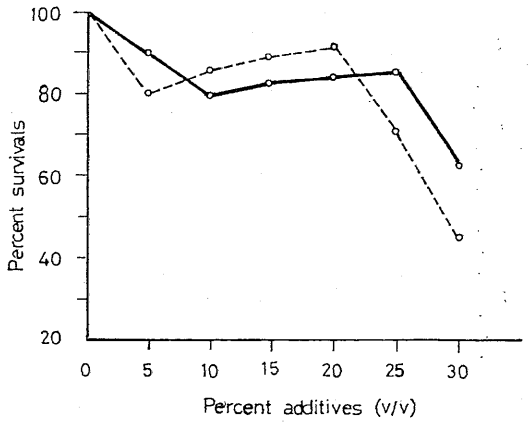


Fig. 2. Toxicity of cryoprotective additives, glycerol (solid line) and DMSO (dashed line), to *Angiostrongylus cantonensis* larvae in DMEM medium.

additives in NCTC 109 medium was chosen as the maximum nontoxic cryoprotectant in further experiment.

Fig. 3 shows the survivorship of thawed larvae after freeze-stored at −15°C for various periods of time. At this temperature, the percentage of recovery first increased with the time, reached to a maximum, and then decreased at longer time periods. The optimum time is 15–20 min. No survival of parasites was detected after thawing when the parasites were cooled directly to −70°C either from 0°C or from room temperature. Fig. 4 summerizes the results of cryopreservation when larvae were deep frozen according to a three-step cooling schedule with 10% DMSO as cryoprotectant at various time intervals of −15°C intermediate temperature. The highest survival rate was obtained when the parasite mixture was held at −15°C for 15–20 min before cooled to −70°C. The further assessment of viability by snail-infection showed that thawed larvae retained their infectivity for susceptable snails after cooling to −70°C. The 15 to 20 min of exposure at the intermediate temperature allowing the highest subsequent infective rate. Twenty percent of the larvae after thawing developed into third-stage larvae in snails and this is comparable to the unfrozen controls by $t$-test ($p>0.05$).

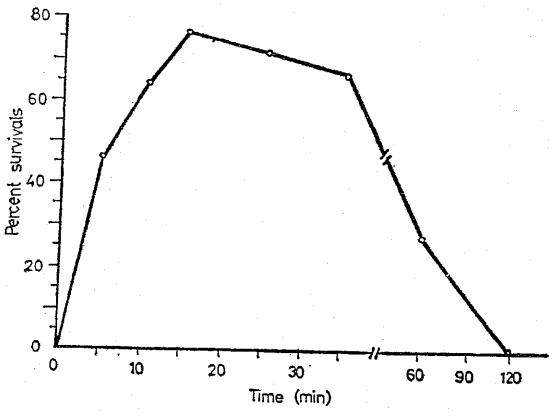


Fig. 3. Survival of *Angiostrongylus cantonensis* thawed larvae held at −15°C for various time intervals.

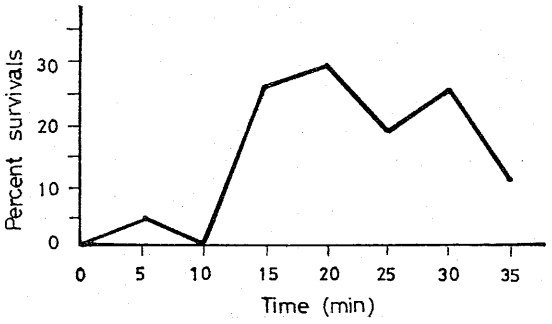


Fig. 4. Survival of *Angiostrongylus cantonensis* thawed larvae following exposure to −15°C for different time intervals then cooled to −70°C for at least 4 days before rewarming.

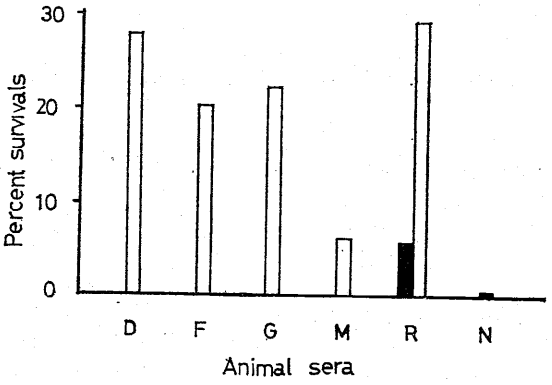


Fig. 5. Survival of *Angiostrongylus cantonensis* thawed larvae after frozen and thawed with various heat-inactivated sera from dog (D), fetal calf (F), goat (G), mouse (M), and rabbit (R). Larvae frozen and thawed with normal rabbit serum (solid bar) and without animal serum (N) are used as controls.

Fig. 5 shows the survival of the first-stage larvae after cryopreservation in the freezing medium prepared with different animal serum additives. The presence of serum is essential for preservation of this parasite. Heat-inactivated serum had better protective effect than serum without heat-inactivation. The survival of larvae recovered after frozen-and-thawed were higher in sera collected from rabbit and dog. Among serum additives which increased larval survivals, monkey serum exhibited the least effect.

## DISCUSSION

The maintenance of parasitic helminths is laborious and costliness. Cryopreservation technology is a better alternative than passages back and forth between laboratory animals and intermediate hosts.

Cryoprotectant additives are often employed during freezing-and-thawing to protect the helminth against irreversible injury which renders the parasite nonviable. However, cryoprotectants are usually toxic and the toxicity is influenced by the concentration and the time and temperature of exposure. The survival of helminth parasites is usually depended on the specific methods of freezing which vary from stage to stage and from species to species. The cryopreservation of nematode parasites has been only successfully achieved in the microfilaria stage of *Brugia pahangi* (Obiamiwe & MacDonald, 1971, Ogunba, 1969), *Dirofilaria immitis* (Bemrick *et al.*, 1965), *Onchocerca volvulus* (Ham *et al.*, 1979, Ham *et al.*, 1981, Schiller *et al.*, 1979), and *Wuchereria bancrofti* (Weinmann & McAllister, 1947), the third-stage larvae of *Haemonchus contortus* (Cambell *et al.*, 1972) and other parasites (Campell & Thomson, 1973), and the egg stage of *A. cantonensis* Uga *et al.*, 1983). Among them, only *W. ban croftican* can be preserved without cryoprotectant additives which are essential for others.

James and Farrant (1977) first employed a two-step cooling schedule to preserve parasitic helminth, *Schistosoma mansoni*, and obtained approximately 0.4% of the organisms survived. Uga *et al.* (1983) modified the method to preserve eggs of *A. cantonensis* and observed a survival rate less than 1%. Compare to other stages in the nematode life cycle, the egg stage seems easier to be cryopreserved because of its relative undifferentiation. Uga *et al.* (1993) also indicated that eggs at the one-cell, 8-cell, 32-cell, and more than 32-cell stages, in addition to pre-embryonated and embryonated eggs had different potential to survive freezing. This is the first reported attempt to cryopreserve first-stage larvae of *A. cantonensis*. Attempts to preserve first-stage larvae in liquid nitrogen (−196°C) were failed in our preliminary experiments and −70°C was chosen as storage temperature in this investigation.

The parallel relationship showed in Figs. 3 and 4 indicated that there must be an optimum condition for period of intermediate temperature. Motility would be decreased when temperature or timing is more or less than this optimum. Similar observations have been reported in the two-step cooling of *Onchocerca* microfilariae by Ham *et al.* (1979). If samples were held at the intermediate temperature for a longer period, a shrunken state would introduce high concentration of intracellular salts. If the incubation period was too short, an incomplete equilibration state would produce intracellular ice which would damage the parasite (Morris & Farrant, 1973, Pribor, 1975). Only the best timing at proper staying temperature would produce the highest survival rate.

The observation of snail-infectivity by cryopreserved first-stage larvae is an important criterion to judge the viability of thawed larvae. The initiation of an infection in snails is primarily dependent on those larvae which exhibiting the least degree of freezing damage. The comparatively short time required for *A. cantonensis* larvae to lose their ability to infect the final host when exposed to a low temperature without cryoprotectant has been investigated by Alicata (1967). The vitality of the third-stage larvae as determined by rat-feeding experiments was destroyed when they were kept at −15°C for only 12 min. In the present study, −15°C was employed only as a temporatory holding temperature for further cooling to −70°C. Damage occurs when larvae were cooled at this temperature for more than 35 min and all larvae died when holding time exceed 2 hr as indicated in Fig. 3. The 15 to 20 min of exposure at the intermediate temperature was determined as optimum

period for the least damage caused by the intermediate and storage temperatures. It is obvious that the technique we developed in this study can improve the preservation of this parasites.

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# 廣東住血線蟲在冰凍溫度下存活之研究

趙大衛　張國禎　黃台珠

本研究探討廣東住血線蟲第一期幼蟲在冰凍溫度下存活之情形，寄生蟲直接由實驗感染之大白鼠糞便中取得。首先在測試二種冰凍保存液毒性時發現二種保存液在使用時均不得高於 30%，而 DMSO 在 NCTC 109 培養液中可容許之最高不致毒濃度爲10%；其次在測定不同動物血清對冰凍幼蟲保護之影響時，發現在 56°C 置過 30 分鐘之去活化兎血清對幼蟲有最高之保護力。因此，本研究中以 NCTC 109 培養液添加 10% DMSO 及 20%去活化兎血清做冰凍保護液來決定最佳之冰凍步驟。結果顯示幼蟲若以此種成份之冰凍液在 0°C 存置五分鐘後移到 −15°C 經 15～20 分鐘，並迅速冰凍到 −70°C 可得到最高存活率，解凍後幼蟲之生物活性並進一步以螺蟛感染力來測試，在 30%解凍存活之幼蟲中有 20%可在扁螺中間宿主體內發育爲第三期幼蟲。