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Improvements in the infectivity of cryopreserved third-stage larvae of Angiostrongylus cantonensis using a programmable freezer

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Abstract Although there have been some advances in the cryopreservation of Angiostrongylus cantonensis, the degrees of viability and infectivity of the cryopreserved developmental stages have not been high. A two-step freezing protocol using a programmable freezer was determined to be effective in improving the infectivity of the cryopreserved third-stage larvae of this parasite. After washing steps and suspension in 10% (v/v) dimethylsulfoxide and equilibrium at room temperature the larvae were placed into the freezer. The temperature was lowered first at 0.8 °C/min from room temperature to −40 °C and then at 10 °C/min to −70 °C. The samples were plunged into liquid nitrogen. After storage in liquid nitrogen for 7–15 days the larvae were thawed rapidly in 37 °C water and 27.6% were found to show vigorous “S-shape” movement without significant changes in appearance. These larvae (50/rodent) could develop to the fifth stage in mice (42.6%) and establish patent infection in rats (40.4%). Moreover, there was no significant difference in the recovery rates of cryopreserved worms and their unfrozen counterparts. These findings indicate that steady precooling conditions may decrease damage with regard to the infectivity of cryopreserved third-stage larvae of A. cantonensis.

Introduction

Angiostrongylus cantonensis is the etiologic agent of human eosinophilic meningoencephalitis in East Asia and South Pacific islands (Hung and Chen 1988). This parasite requires a mollusc intermediate host and a rodent definitive host for completion of its life cycle. Although we have maintained the life cycle of A. cantonensis in our laboratories for many years, the collection of sufficient living worms of the same developmental stage remains an important technical problem in our research. Recent advances in cryobiology have shed light on our problem, since reliable cryopreservation techniques can facilitate the storage of a considerable number of living helminths. In addition, these techniques also preserve the antigenic, pathogenic, and other biologic characteristics of the worms (James 1985).

In recent years there has been some progress in the cryopreservation of A. cantonensis (Uga et al. 1983; Chao et al. 1988, 1993; Mahajan and Renapurkar 1993). Although the thawed larvae had a high degree of viability, their level of infectivity was not high (Chao et al. 1993; Mahajan and Renapurkar 1993). Since the programmable freezer has been demonstrated to be a useful tool in successful maintenance of the infectivity of cryopreserved third-stage nematode larvae (James and Peacock 1986; Andermatt-Mettler et al. 1987; Nolan et al. 1988), in the present study we attempted to improve the infectivity of cryopreserved third-stage larvae of A. cantonensis using this machine.

Materials and methods

The life cycle of Angiostrongylus cantonensis has been maintained in our laboratory by cycling through Biomphalaria glabrata snails and Sprague-Dawley (SD) rats since 1980 (Wang et al. 1989). Third-stage larvae were isolated from the infected snails and washed twice with normal saline. For each replicate, larvae were pooled from a single batch of snails.
Toxicities of glycerol and dimethylsulfoxide (DMSO) were assessed by incubation of 100 third-stage larvae with test solutions (2 ml) containing each compound and RPMI 1640 medium (Gibco Laboratories, Life Technologies, Inc., Grand Island, N.Y., USA) at different ratios (0–50%, v/v). After incubation for 30 min at room temperature, 10 ml of normal saline was added to the test solution and the parasite suspension was centrifuged at 900 g for 7 min. The larvae were then washed twice with normal saline and resuspended in 10 ml of RPMI 1640 medium. Toxicities of the cryoprotectants were determined by the counting the number of motile larvae under a dissecting microscope 30 min later. Three replicates were performed for each cryoprotectant.

After washing steps and suspension of larvae in a solution containing RPMI 1640 medium, the cryoprotectant, and fetal calf serum an aliquot of 50 third-stage larvae was transferred to a cryotube. Each aliquot was subjected to one of four freezing protocols. The frozen larvae were then stored in liquid nitrogen for 7–15 days.

The freezing process began after equilibrium for 30 min at room temperature. In protocol A the larvae were first placed in a 4 °C refrigerator for 5 min and then stored in a −20 °C freezer for 10 min. After the second freezing step the larvae were transferred to a −70 °C freezer for 30 min. In protocol B the larvae were transferred to the −70 °C freezer for 10 min after storage in the 4 °C refrigerator for 5 min. A programmable freezer (IceCube 1610 Computer Freezer, SY-LAB, Austria) was used in protocols C and D. In protocol C the temperature was lowered first at 0.8 °C/min to −40 °C and then at 10 °C/min to −70 °C. In protocol D the temperature was programmed to decrease at 0.8 °C/min to 0 °C. After equilibrium for 15 min, the temperature was then lowered first at 0.8 °C/min to −40 °C and then at 10 °C/min to −70 °C. After the precolling step, all samples were plunged into liquid nitrogen.

The frozen larvae were thawed rapidly by immersion and shaking of the cryotubes in 37 °C water for 2 min. The suspensions were immediately diluted with normal saline. The larvae were then washed with 10 ml of normal saline and centrifuged twice at 900 g for 7 min.

The survival of the thawed larvae was assessed by examination of their motility and infectivity. Gross morphology and motility were observed under a dissecting microscope at 30 min after thawing. Those showing “S-shape” movement and no significant change in appearance were considered normal. Abnormal larvae for 7 min.

Infectivity tests were conducted using the normal thawed third-stage larvae and their unfrozen counterparts in male BALB/c mice (8 weeks old) or SD rats (8 weeks old). Each animal was inoculated with 50 larvae through stomach intubation. The infected mice were killed 14 days after infection and the numbers of fifth-stage larvae (8 weeks old) or SD rats were killed at 50 days post-infection and the adult worms in their brains were determined. Patent infections in rats were assessed by the presence of first-stage larvae in the feces. The infected rats were killed at 50 days post-infection and the adult worms collected from the heart and lungs were counted.

### Results

The results of the toxicity assay are shown in Fig. 1. No larva was viable in the group frozen according to protocol A. In protocol B, only 8% of the cryopreserved larvae were normal after thawing. However, higher survival rates were obtained when the programmable freezer was applied in the freezing process (protocol C 27.6%, protocol D 29.9%). Because of the limitations in materials and facilities, experiments on the infectivity of thawed larvae in mice and rats were conducted only in the group frozen according to protocol C.

The cryopreserved third-stage larvae could develop to the fifth stage in BALB/c mice and establish patent infection in SD rats. Moreover, there was no significant difference in the worm recovery rates recorded for the cryopreserved larvae and their unfrozen counterparts (Students t-test, P > 0.05; Tables 1, 2).

### Discussion

Although Uga et al. (1983) have succeeded in preserving *Angiostrongylus cantonensis* eggs at a low temperature (−196 °C), the survival rate of cryopreserved eggs was

### Table 1 Recovery of fifth-stage larvae from BALB/c mice after infection with cryopreserved or unfrozen third-stage larvae of *Angiostrongylus cantonensis*

<table>
<thead>
<tr>
<th>Larvae</th>
<th>Number of mice</th>
<th>Worm recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryopreserved</td>
<td>10</td>
<td>42.6 ± 6.1</td>
</tr>
<tr>
<td>Unfrozen</td>
<td>10</td>
<td>44.8 ± 5.2</td>
</tr>
</tbody>
</table>

*a* Each mouse was infected with 50 third-stage larvae

*b* Larvae were stored in liquid nitrogen for 7 days and frozen by a two-step protocol using a programmable freezer at a rate of first 0.8 °C/min from room temperature to −40 °C and then 10 °C/min to −70 °C.

**c** Student’s t test: t = 0.8681, P > 0.05.

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![Fig. 1 Survival rates of third-stage larvae of *Angiostrongylus cantonensis* in DMSO (solid line, ◦) and glycerol (broken line, ○) as determined at different concentrations](image-url)