

# SURVIVAL AND INFECTIVITY OF *BRUGIA MALAYI* MICROFILARIAE AFTER CRYOPRESERVATION

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**Abstract.** Methods were studied for the cryopreservation of microfilariae of periodic *Brugia malayi*. RPMI-1640 tissue culture medium containing 6% dimethyl sulfoxide (DMSO) and 15% newborn calf serum was used as cryoprotectant. Samples were frozen slowly in the vapor phase of liquid nitrogen prior to emersion in liquid nitrogen (-196°C). The freezing rate was -0.5 to -1.0°C per minute, microfilariae remained viable for as long as, 212 and 375 days, survival rates were 94 to 98% and they were infective to *Aedes togoi* mosquitos.

The infective larvae (L<sub>3</sub>) were obtained for 10-11 days after feeding at 28°C room-temperature and the infection rate of L<sub>3</sub> in test mosquitos was 22.4-30.6%. All DMSO should be removed from the freezing medium to restore microfilariae activity after freezing.

## INTRODUCTION

Deep-freezing and storage in liquid nitrogen is a well-established and widely used technique for preserving filarial parasites in a viable state (McCall *et al*, 1975; Owen and Anataraman, 1982; Ham and James, 1982; Ham and Townson, 1986; Lowrie, 1983). These techniques allow the preservation of viable parasites and establishment of parasite banks for living reference material. This study demonstrates that microfilariae (Mf) of *Brugia malayi* can be frozen and stored for extended periods. The present report on the cryopreservation in liquid nitrogen of Mf is of a periodic strain of *Brugia malayi* from Guizhou, China.

## MATERIALS AND METHODS

The periodic strain of *B. malayi* was established in Mongolian jirds (*Meriones unguiculatus*) in this laboratory. Mf were collected from the abdominal cavity of infected jirds washed extensively three times in sterile saline and centrifuged at 1,500 rpm for 10 minutes. The Mf were suspended at about 5,000 Mf/ml of cryoprotectant consisting of RPMI-1640 tissue culture medium (pH 7.2) with 15% newborn calf serum and 6% DMSO. Suspensions of Mf were transferred to 2ml ampoules. Three methods were used for the cryo-

preservation of Mf:

1. Specimens were put down to a depth of 15 cm in the mouth of the liquid nitrogen container for one hour, the temperature gradually decreased 0.5°C to 0.8°C per minute (to about -50°C). Then the samples were placed 5 cm lower down for one hour (about -70°C to -90°C). They were then plunged directly into the liquid nitrogen (-196°C).

2. Specimens were frozen at a uniform rate of -0.5°C to -1.0°C per minute using the vapor phase of liquid nitrogen; when the temperature reached -70°C to -90°C, they were placed directly into the liquid nitrogen and stored at -196°C.

3. Specimens were incubated at 4°C for two hours then, as described in method 1, put down into liquid nitrogen.

Mf were stored for 6, 212 and 375 days in cryogen. Ampoules were thawed rapidly by immersion in a water bath at 37°C for one minute. Then 1ml of the thawed contents were put in a 10ml centrifuge tube and 9ml of warm (37°C) sterile saline added. The samples were washed 3 times by centrifuging at 1500 rpm for 10 minutes and discarding the supernatant. The washing procedure is required to eliminate DMSO which is toxic.

Viability of thawed Mf was assessed on the

basis of motility and survival. Aliquots of suspension containing the Mf were transferred to concave slides and examined under a microscope. 100 Mf were examined for morphology, motility noted and the survival rate assessed using methods described by Tao and Huang (1986). Mean activity scores were calculated for 100 of Mf and were compared using Chi-square analysis for thawed and control Mf.

The following criteria were used to score the activity:

- 0 degree = inactive or dead Mf
- 1 degree = very sluggish or intermittent movement
- 2 degree = slowly movement
- 3 degree = middle movement
- 4 degree = constant, vigorous serpentine movement (normal Mf)

$$\text{Survival rate} = \frac{\text{No. living Mf}}{\text{Total No. of Mf (living or dead)}} \times 100$$

Laboratory-bred *Aedes togoi* were used as the intermediate hosts for *B. malayi*. Thawed Mf were mixed with defibrinated fresh rabbit blood and the volume adjusted to give 180-208 Mf/per 20mm<sup>3</sup> of blood. The blood was placed in a placental membrane feeder apparatus. They were placed at room temperature (28°C) and fed a 10% sugar solution soaked in plastic pads. The infective larvae (L<sub>3</sub>) were obtained 10-11 days after feeding.

Because DMSO is toxic to cells, in order to select the best concentration for frozen Mf 1 to 20% concentrations of DMSO respectively were tested in frozen storage of Mf for two weeks.

### RESULTS AND DISCUSSION

Table 1 lists the survival and activity scores of Mf of *B. malayi* at various times after cryopreservation in liquid nitrogen. After thawing, 94 to 98% of the total Mf of all samples appeared to

Table 1

The relation between the time and viability of Mf of *Brugia malayi* after cryopreservation.

Object of experiment	Control (unfrozen)	Method 1			Method 2			Method 3		
		Days frozen			Days frozen			Days frozen		
		6	212	375	6	212	375	6	212	375
Survival of Mf	99	96	95	97	95	96	98	94	97	98
Activity score of Mf	4	3.3	2.1	3.3	3.3	3.2	3.3	2.4	2.5	2.2

Table 2

The relation between time of cryogenic storage and development of Mf *Brugia malayi* in *Aedes togoi* mosquitos.

Days frozen	Survival rate of L <sub>3</sub> (%)	Activity* score of L <sub>3</sub> (x̄)	Density of Mf feed test mosquitos (20 mm <sup>3</sup> )	Infection rate of L <sub>3</sub> in test mosquitos (%)	No. L <sub>3</sub> test mosquitos	
					L <sub>3</sub> No./per mosquito	Mean
Control	100	1.9	208	36.3 (57/157)	1-12	4.2
6	100	1.8	198	22.4 (57/255)	1-4	1.3
212	98	1.7	180	25.0 (8/32)	1-3	2.0
375	99	1.9	236	30.6 (15/49)	1-6	2.46

\* The method was as described by Lok *et al* (1983).

0 degree = Inactive or damaged third-stage larvae.

1 degree = Sluggish or intermittent.

2 degree = Constant, vigorous serpentine movement (normal larvae).

have survived and were moving normally.

There was no significant difference found between unfrozen Mf and frozen Mf with respect to survival rate ( $p > 0.05$ ), while the activity score was 2.1 to 3.3 lower in frozen than unfrozen Mf.

Using Mf thawed at various times after cryopreservation in liquid nitrogen were fed to *Aedes togoi* as the intermediate host for *B. malayi*. The results showed survival rates and activity scores of L<sub>3</sub> from 98 to 100% and 1.7 to 1.9 respectively (Table 2).

The larval morphology and mobility were the same as those of the control. Also the survival rate and activity score of L<sub>3</sub> from test mosquitos were the same as the control ( $p > 0.05$ ), but the infection rate and the number of L<sub>3</sub> in the test mosquitos were lower than controls (unfrozen).

The test results indicated a concentration of DMSO used in cryoprotectant of 4 to 7 percent afforded the best cryoprotective effect. The survival rate of Mf was 100%. At a concentration of DMSO of 13% about 10% Mf died and the death rate of Mf gradually rose when the concentration of DMSO increased. At 20% DMSO, 90% of Mf were dead. When the concentration of DMSO was 1 to 3%, the mortality rates of Mf were 1.6%, 1.1% and 1.1% respectively.

Our experiments have shown that microfilariae of the Guizhou strain of *B. malayi* can be frozen and stored for extended periods without impairing infectivity and survival rate when specimens were stored in liquid nitrogen (-196°C), but the freezing rate in the vapor phase of liquid nitrogen needs to be carefully controlled. There was no correlation between the length of cryogenic storage and the recovery of the infective larvae in *Aedes togoi* mosquitos.

Freezing the samples too slowly or too quickly resulted in death of the larvae because of ice crystal formation or cell water loss, raising the dielec-

tric concentration (Ham *et al*, 1979). In the present study, gradually chilling the sample in vapor phase before immersion directly into the liquid nitrogen was used, the survival rate was optimized at a freezing rate of -0.5°C to -1.0°C per minute. In addition, it has been pointed out that after thawing DMSO must be removed from the freezing medium in order to restore normal microfilariae motility and infectivity.

Our results suggest that the rate of freezing in the vapor phase of liquid nitrogen and the concentration of DMSO were very important.

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