Method for Storing *Toxoplasma gondii* (RH Strain) in Liquid Nitrogen

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A simple method for storing the trophozoites of *Toxoplasma gondii* (RH strain) is described. Viability of the parasite was maintained for 160 days.

Refrigeration with liquid nitrogen (LN₂) at −196°C has given parasitologists and other workers a useful and satisfactory method for long-term storage of parasites and other biological material. There are two main advantages in storing *Toxoplasma gondii* for prolonged periods. (i) Serial passage of the parasite in mice is eliminated, along with the associated hazard of that procedure. Even with careful techniques, cases of laboratory-contracted toxoplasmosis still occur (3). (ii) Strains of toxoplasmosis can be preserved at various stages in experiments, and the original infectivity and antigenicity of the parasite can be maintained.

Protozoa require cryoprotective agents when refrigerated at low temperatures (6), and a controlled freezing rate is recommended (1), although the latter does not appear to be critical for the survival of some *Plasmodia* (5). Glycerol was used successfully as a cryoprotectant by Eyles and co-workers (2). In this laboratory the cryoprotectants, glycerol and dimethyl sulfoxide (DMSO), when used separately and in combination at concentrations ranging from 2.5 to 20%, gave unsatisfactory results. No survival of the organism was noted after freezing and storage in liquid nitrogen for 8 days. Preservation was also poor when similar concentrations of glycerol and DMSO were combined with 20% fetal calf serum. These experiments were performed with controlled freezing at 1°C/min from room temperature to −70°C, followed by immersion directly into LN₂.

A simple and successful technique was developed for long-term storage and survival of *T. gondii* trophozoites (RH strain) with fetal calf serum as the cryoprotectant. Controlled freezing as described was not found necessary and in fact seemed detrimental to the survival of the parasites.

*T. gondii* (RH strain) was obtained from Walter Stahl of this Division as a saline suspension of trophozoites from the peritoneal exudate of adult mice (Albany white NYLAR-A strain). The parasite was readily adapted to cultures of diploid human embryo lung (HEL) cells, grown and maintained on Eagle medium with 5% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and mycostatin (25 μg/ml). With this method, the parasite at the 56th passage in cell culture remains lethal for mice when injected intraperitoneally, and contamination with bacteria and mouse peritoneal cells and fluid is avoided.

Protozoa from the 8th passage in HEL cells were used. The trophozoites were harvested when more than 90% of the cells showed cytopathic changes. The cells and fluid were centrifuged at 1,400 × g for 10 min in a nonrefrigerated centrifuge. The supernatant fluid was discarded, and the sediment was resuspended in undiluted fetal calf serum which had been

### Table 1. Survival of the trophozoites of *Toxoplasma gondii* (RH strain) before and after storage in LN₂

| Time          | Viable no. of trophozoites (per ml) | LD₅₀ titer for mice | Highest dilution showing cytopathic effects in HEL cell culture |
|---------------|------------------------------------|--------------------|---------------------------------------------------------------|
| Before freezing | 1.2 × 10⁷                          | 10⁻⁷               | 10⁻⁴                                                         |
| After 60 days in LN₂ | 0.9 × 10⁴                          | NT                 | 10⁻⁴                                                         |
| After 100 days in LN₂ | 1.4 × 10⁴                          | 10⁻⁶               | 10⁻⁵                                                         |
| After 160 days in LN₂ | 1.1 × 10⁵                          | 10⁻⁷               | 10⁻⁴                                                         |

*Total counts performed by using hemocytometer and trypan blue staining method (4).  
LD₅₀ (mean lethal dose) titer by the Reed-Muench method. Mice (10 to 12 g, Albany white strain) were inoculated intraperitoneally.  
Flasks (250 ml) of HEL tissue inoculated with 7.5 ml of diluted material.  
NT, Not tested.
stored at 4 to 10 C. This serum was free of toxoplasma antibodies as assessed by the indirect hemagglutination test at a 1:10 dilution.

The suspended trophozoites were dispensed in 1.5-ml amounts into plastic ampoules with screw caps (Pro-Vials, Microbiological Associates, Bethesda, Md.). Autoclave tape (3M Co., Philadelphia, Pa.) and a ball-point pen were used for labeling. The ampoules were immersed in LN2 within 30 min. No breakages or explosions occurred.

The parasites were retrieved from the frozen state by immersing the ampoules in a 37 C water bath. A slight buildup in pressure in the ampoule was observed, and, to avoid aerosols when opening, the cap was slowly released with the entire ampoule held wrapped in absorbent cotton soaked in antiseptic.

The trophozoite suspension was used immediately after thawing for the inoculation of cell cultures and mice and for vital staining.

Table 1 shows the survival of T. gondii (RH strain) as determined by three different assays. Eyles and his co-workers (2) were able to store an unspecified strain of T. gondii at below -70 C for 209 days. By using 5% glycerol as the cryoprotective agent, they found a $10^{3.57}$ reduction in titer during freezing, but during storage little further loss of viability occurred. The method described in this report showed a loss of only 1 log of viable parasites during freezing as determined by the trypan blue staining method and the titration in mice. The number of viable parasites remained constant over a 160-day storage period, and it seems likely that infectivity will be preserved for a longer period.

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