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Cryopreservation of parasites

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Summary. In this review, advances in cryopreservation of helminth parasites are reported. Our own studies demonstrate that metacestodes of *Echinococcus multilocularis* can be maintained in a viable state for at least 1–2 years by appropriate deep-freezing and storage in liquid nitrogen. Infective larvae of the nematode *Toxocara canis* cryopreserved for 1 week in liquid nitrogen were maintained after thawing in vitro in a chemically defined medium for 35 weeks. Although motility of previously deep-frozen larvae was reduced they produced secretory/excretory antigens of similar immunodiagnostic quality as those from unfrozen larvae. Whereas infective larvae of several species of trichostrongylids can be easily cryopreserved, the infective larvae of the cattle lungworm, *Dictyocaulus viviparus*, and muscle larvae of *Trichinella spiralis* are more sensitive to damage by subzero temperatures. Therefore, survival rates after cryopreservation are low, but improvement of the cooling schedules appears to be feasible. It is concluded that cryopreservation of certain stages of helminth and protozoan parasites is a useful technique for long-term storage of defined isolates, which can contribute considerably to reducing the number of experimental animals usually required for serial passages.

Key words. Cryopreservation; *Echinococcus multilocularis;* trichostrongylids; *Toxocara canis; Dictyocaulus viviparus; Trichinella spiralis;* alternatives to animal experimentation.

Introduction

Deep-freezing and storage in liquid nitrogen is a well-established and widely used technique for preserving protozoan parasites in a viable state[12, 16, 17, 22, 27–30]. In contrast, the application of this method to the metazoan helminths is still limited[12], although several species and stages have been cryopreserved successfully, for example the infective larvae of several species of trichostrongylids and strongylids[2, 4, 31, 34, 36], the microfilariae and/or infective larvae of *Brugia, Dirofilaria, Dipetalonema, Litomosoides, Wuchereria* and *Onchocerca*[9, 10, 11, 25, 26] and the sporocysts and schistosomula of *Schistosoma mansoni*[3, 13, 14, 18, 19, 23, 24, 35].

Reliable cryopreservation techniques for parasite stages can contribute considerably to the reduction of the number of experimental animals needed for strain maintenance in laboratories. In addition, these techniques would allow the preservation of viable parasite isolates and strains with interesting characteristics (drug resistance, high or low antigenicity etc.), and the establishment of parasite-banks for living reference material, and they would also facilitate storage for long periods and transport between laboratories[5, 6, 16]. Further, they could be applied in the production of live vaccines as shown by James and Dobinson[17] and Lewis et al.[23, 24] with schistosomula of *S. mansoni.*

In the following report we summarize some results of our recent research activities on cryopreservation of helminth parasites, which have been published in extenso elsewhere[1, 7, 32, 33].

Experiments and results

1. Cryopreservation of Echinococcus multilocularis metacestodes

The larval (metacestode) stage of *Echinococcus multilocularis*, which causes alveolar echinococcosis in humans, is

maintained in laboratories for research purposes by serial passages in rodents (jirds, cotton rats and mice, etc.). In the highly susceptible jird (*Meriones unguiculatus*) passages are necessary every 2–5 months, depending on the dose and mode of infection as well as the characteristics of the parasite 'isolate'. Therefore, the maintenance of larval *E. multilocularis* requires many experimental animals which may suffer from the infection because of the tumor-like proliferation of the parasite.

In order to reduce the number of animals needed for parasite strain maintenance and to elaborate reliable techniques for the preservation of 'stabilates' with defined characteristics allowing the storage of reference material of viable parasites, studies on cryopreservation of the metacestode stage of *E. multilocularis* were carried out by Eckert and Ramp [7].

Four isolates of larval *Echinococcus multilocularis* originating from Switzerland (CH/1, CH/6 and CH/22) and Alaska (A/1) were used to prepare crude homogenate or small tissue fragments (STF) in Eagle's Minimal Essential Medium with Earle's salts (EMEM/A), or 0.2 g tissue blocks (TB) which were suspended in the same medium. After addition of dimethylsulfoxide or glycerol in final concentrations of 5% and 10% (v/v), respectively, aliquots of 1.0 ml, containing either 0.1 ml crude homogenate or STF, or one block of 0.2 g, were kept in cryotubes for 30 min at +2–4 °C (precooling phase), cooled subsequently to lower temperatures following a two-step or three-step schedule and finally plunged into liquid nitrogen (−196 °C). After storage for one week the samples were rapidly thawed at +37 °C for approximately 3 min, washed in fresh EMEM/A (37 °C) and transferred into the peritoneal cavity of *Meriones* for viability testing. As judged by histological examinations and metacestode weights of each of the 24 *Meriones* infected with cryopreserved homogenate, STF or TB, respectively, 46%, 87% or 100% of the animals contained viable, proliferating parasites. The best proliferation rate occurred when 10% glycerol was used as cryoprotectant and after precooling a three-step freezing schedule was employed (30 min at −28 °C, 30 min at −80 °C, transfer to liquid nitrogen). Cooling rates were determined as 0.7, 1.0 and 1.7 °C min^{-1} for the precooling phase, step 1 and step 2, respectively, and estimated as 65 °C min^{-1} for step 3. These results demonstrate that metacestodes of *E. multilocularis* can be successfully maintained by cryopreservation without losing their proliferative capacity in the intermediate host. An example is presented in the table [7]. Further experience gained since the publication of our first results [7] indicates that *E. multilocularis* metacestode material can be cryopreserved in a viable stage for at least 1 to 2 years. Therefore, most of the *E. multilocularis* metacestode isolates in our laboratory are now maintained by cryopreservation and they are only transferred to animals if this is necessary for experiments.

2. Cryopreservation of infective larvae of trichostrongylids

Trichostrongylids are important nematodes inhabiting the gastrointestinal tract of cattle, sheep, goats, wild ruminants, several other animal species and occasionally also of humans. The adult female parasites produce eggs which are excreted by the parasitized hosts with the feces. Outside the host first, second and finally third-stage larvae develop from the eggs. The third-stage larvae are protected by a cuticular 'sheath' and act as infective stages which are ingested by the animals with contaminated plants.

The infective larvae of certain species of trichostrongylids can overwinter on pasture and they may also resist freezing for limited periods. For example, 0.1% of the third-stage larvae of *Ostertagia ostertagi* can survive at −10 °C for 43 days, but at −25 °C all larvae are killed within 78 h [21]. This example indicates that special procedures may be required for the successful cryopreservation of these larval stages. Several benefits can be gained from cryopreservation; these include a considerable saving of the animals, time, labor and other costs involved in continually maintaining monospecific isolates in animals, and the possibility of preserving drug resistant and genetically homogeneous material.

Cryopreservation of third stage trichostrongylid larvae is relatively easy to perform, as indicated by several authors

Viability of larval *Echinococcus multilocularis* in *Meriones* after cryopreservation of metacestode tissue blocks for one week in liquid nitrogen (Experiments No. G831[CH/1], G835[CH/6] and G839[CH/22])

Parasite isolate	Freezing schedule[a]	Cryoprotectant[a]	Viability test in *Meriones* (intraperitoneal infection) No. animals infected	No. animals with metacestodes	Metacestode weight (g) $\bar{x}$ per animal	Range	Increase[b]	Histology[c] C	P	PS	Viability[d]
CH/1	3-step	glycerol	4	4	4.5	2.5–6.2	22.5×	+	+	+	viable (4)
CH/6	3-step	DMSO	5	4	0.9	0.2–1.1	4.5×	+	+	+	viable (4)
	3-step	glycerol	5	5	3.1	0.1–6.8	15.5×	+	+	+	viable (5)
CH/22	3-step	DMSO	5	4	1.0	0.2–3.3	5.0×	+	+	+	viable (4)
	3-step	glycerol	5	4	2.2	1.5–3.9	11.0×	+	+	+	viable (4)
	control[e]	none	4	4	17.2	10.4–30.3	85.9×	+	+	+	viable (4)

a) For freezing schedule and cryoprotectant see text; b) Dose of infection per animal: 0.2 g (× = times); c) C: cysts; P: protrusions; PS: protoscolices; +: present; d) Parasite viability; numbers in parenthesis indicate number of animals. e) Control: untreated tissue blocks of the three isolates were transplanted to *Meriones* for routine strain maintenance and showed normal proliferation (quantitative determination in the case of CH/22 only). (after Eckert and Ramp, 1985) [7].

in previous years[32]. An important prerequisite for successful cryopreservation is the removal of the protective 'sheath' from the larvae by treatment with sodium hypochlorite solution[32].

In our experiments[32] exsheathed third-stage larvae of *O. leptospicularis* and of two isolates of *Haemonchus contortus* were suspended in phosphate buffered physiological saline (PBS) and frozen in the gas phase of liquid nitrogen at an average cooling rate of 9.9 °C/min. The frozen larvae were then stored for 16 weeks in liquid nitrogen. After thawing, the larvae were perorally transmitted to 6 helminth-free sheep. All the animals developed patent infections.

These results are in agreement with findings of other authors[32] that cryopreservation is an easy and feasible technique for long-term storage of defined isolates of trichostrongylid larvae. It has been shown[34] that the third-stage larvae of *H. contortus* were still infective for animals after 10 years of cryopreservation. Therefore, this procedure can contribute to reducing the numbers of experimental animals.

3. Cryopreservation of Toxocara canis larvae

Toxocara canis, an ascarid nematode and a common intestinal parasite of dogs, can also infect man and domestic animals causing a disease known as 'larva migrans interna'. Infection occurs by the ingestion of second-stage larvae enclosed in the egg-shell. After infection, the larvae liberated in the intestine migrate to various organs, such as liver, lung, central nervous system and eye, and may cause the formation of granulomatous lesions. The second-stage larvae can be isolated from developed eggs and may be maintained in a chemically defined medium for about one year. Excretory/secretory antigens released by the larvae in vitro are used for sero-diagnosis in the ELISA, i.e. for the detection of antibodies against *Toxocara* in sera of humans and animals.

With the aim of establishing a 'bank' of viable *Toxocara* second-stage larvae studies on cryopreservation of such larvae were carried out[33].

Second-stage larvae of *Toxocara canis* were isolated from developed eggs, frozen in Eagle's Minimal Essential Medium with 5% dimethyl sulfoxide or 10% glycerol as cryoprotectants according to two cooling schedules, and maintained in liquid nitrogen for 1 week. After thawing, the previously frozen larvae (FL) and unfrozen controls (CL) were maintained in a chemically defined medium in vitro for 35 weeks. While CL had motility rates around 95%–97% throughout the experiment, previously frozen larvae (FL) exhibited rates of 48%–58% at the beginning and of 19%–39% at the end of the 35-week in vitro maintenance period. The surviving FL and CL larvae proved to be infective for mice. Excretory/secretory (ES) antigens isolated from several batches of culture medium in which FL and CL had been maintained reacted in the ELISA with human sera containing antibodies against *Toxocara*. Antigens from FL and CL separated by SDS-PAGE and silver-stained showed some differences in polypeptide patterns. Western-blot analysis revealed that these differences were not related to antigenic polypeptides but were most likely caused by substances without antigenic properties originating from dead and/or degenerating larvae. It can be concluded that ES antigens produced by previously frozen larvae are essentially the same as those derived from unfrozen controls.

The value of cryopreservation of *T. canis* larvae for routine production of ES antigens should be further evaluated.

4. Cryopreservation of Dictyocaulus viviparus larvae

Dictyocaulus viviparus is a nematode species infecting the respiratory tract of cattle, causing 'lungworm disease'. The life cycle of this parasite is a direct one like that of trichostrongylids (see section 2), and the third-stage larvae are infective for the hosts.

Cattle can be vaccinated against lungworm disease by the oral application of a vaccine containing living, radioattenuated third-stage larvae of *D. viviparus*. Although of high practical value, the vaccine has the disadvantage of a very short life-span of about 2 weeks. Therefore, it would be beneficial to develop a method for cryopreserving the larvae in a viable and immunogenic state. Another motivation for developing a cryopreservation schedule for third-stage larvae of *D. viviparus* is the fact that for vaccine production and other research purposes the parasites have to be maintained by frequent serial passages in animals. The infected calves may suffer from lung disease.

Only a few studies have been carried out on the cryopreservation of *Dictyocaulus viviparus* larvae[15, 20]. These studies indicated the difficulty of cryopreserving third-stage *Dictyocaulus* larvae without loss of infectivity to cattle.

In our own studies[1] with third-stage larvae of *D. viviparus*, the following results were obtained:

Best results were achieved by incubating larvae in 0.05% sodium hypochlorite solution at 37 °C to remove the sheath, followed by cooling at a rate of 1 °C min^{-1} down to about 0 °C. After an equilibration time of 10 min at +4 °C with or without 4% polyethylene glycol-400 as cryoprotectant, samples were frozen to an intermediate temperature of −20 °C, maintained at this temperature for 10 min and finally plunged into liquid nitrogen for storage. For viability testing three groups of 3 calves were infected with the following batches of third-stage larvae: (a) fresh, sheathed; (b) fresh, exsheathed; (c) cryopreserved for 13 weeks in liquid nitrogen and subsequently thawed. Although 62% of the larvae in group (c) were regarded as viable in vitro, their infectivity for calves was low and only an average of 0.08% of the inoculated larvae (3000 per animal) developed into adult lungworms (= infectivity rate). Average infectivity rates of fresh, sheathed (a) and fresh, exsheathed (b) larvae were much

higher (38.3% and 29.7%) and not significantly different from each other. Two of the calves inoculated with previously frozen larvae and all of the calves infected with fresh larvae excreted first-stage larvae in their feces, but the latter group in higher quantities. The results show that cryopreservation of exsheathed third-stage larvae of *D. viviparus* is possible, but for strain maintenance infection doses greater than 3000 larvae should be used for inoculation of calves.

5. Cryopreservation of Trichinella spiralis larvae

Trichinellosis is a zoonotic infection of humans and a large number of vertebrate animals species. Disease may be caused by the adult stages inhabiting the small intestine and/or by larvae invading the muscle cells (muscle larvae), where they are encapsulated in a surviving host. Infection of a host occurs after the ingestion of meat containing infective muscle larvae of *Trichinella*.

The aim of our studies with muscle larvae of *T. spiralis* was to evaluate the possibility of maintaining these larvae outside a living host animal in a viable state [1].

In our studies [1] it could be demonstrated that the conditions for cryopreservation of *Trichinella* muscle larvae differ from those reported in the previous chapters. Experiments on applying cooling schedules similar to those used for larval stages of *Echinococcus multilocularis*, various trichostrongylid species, *Dictyocaulus viviparus* and *Toxocara canis* have failed. However, it was possible to preserve these larvae in small blocks of muscle tissue (0.2 g) after addition of undiluted ethylene glycol as cryoprotectant by storage at −10 °C for 12 weeks. About 60% of the thawed larvae were viable in vitro and were still infective for rats. However, the recovery rates of larvae from rats were relatively low.

Conclusions

The examples described above indicate that cryopreservation of infective stages of *Echinococcus multilocularis* and various nematode species is essentially possible. In the case of *E. multilocularis* the cryopreservation technique is so far advanced that it can already be applied in practice, leading to a considerable reduction of numbers of experimental animals. The same applies for the third-stage larvae of trichostrongylids. For other nematode species mentioned above, improvement of cryopreservation techniques is required. Further, it can be anticipated that cryopreservation of infective stages of many other metazoan parasite species may also be feasible. However, the appropriate cooling schedule has to be worked out for each species. This will require further, intensified research activities.

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Short Communications

The use of microtiter plates for the simple and sensitive determination of insulin by an ELISA method

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Summary. A method of insulin determination using a commercially available ELISA kit was modified for use in microtiter plates. The adapted assay, based on the binding of porcine anti-guinea pig insulin antibodies to microtiter plates and insulin-peroxidase conjugate as displacer, is sensitive between 0.5 and 30 ng/ml. Since it uses only 10–40 µl of sample material it enables the determination of 5–100 pg of insulin. The rapid (5–6 h), automatable, reproducible and reliable assay makes it possible to determine many samples in a short time.
Key words. ELISA; insulin determination; peroxidase; microtiter plates.

A number of methods are currently available for the determination of immunoreactive insulin (IRI) in biological samples. The most widely used methods are radioimmunoassays, utilizing ^{125}I-insulin as tracer and a variety of methods for the separation of bound from free insulin[1–3].

A new method, the Enzymun-test® insulin[4], was recently made commercially available by Boehringer-Mannheim. This method relies on the competition between insulin-peroxidase conjugate and insulin in binding to insulin-antibodies coated onto plastic tubes, and a colorimetric determination of the bound peroxidase conjugate. We have adapted and modified this assay for the use in microtiter plates. By using microplate technology and this modified ELISA system hundreds of samples could be cost-effectively determined in a very short time. This modification may enable clinical and research laboratories to determine human or rat insulin easily by utilizing readily available, automatable systems while avoiding the problematic use of isotopes.

Materials and methods. Preparation of plates. For the routine determination of insulin, round-bottom microwell plates (Microwell module U-16 Nunc, immuno quality 80 PCS, elevated absorption capacity) were used. Anti-porcine insulin guinea pig serum (Novo Biolabs M8309) was diluted with 45 ml distilled water to give a final dilution of 1:9000. 100 µl of the antibody solution was pipetted (using a 12 channel pipette) into the wells. The plates were evaporated to dryness at 28 °C for 24–30 h in a Dynatech microtiter incubator (under circulating air). Once dryed, the plates were sealed and stored at 0–4 °C until used.

Assay. 10–40 µl samples were deposited in the wells and 200 µl of phosphate buffer 40 mM pH 6.8 including 0.25% BSA (RIA grade, Sigma) were added. Plates were covered and samples were incubated 2 h at 37 °C in a Dynatech microtiter incubator. Following the incubation, the samples were withdrawn and the plates washed once with 250 µl cold (0–4 °C) tap water. 100 µl of insulin-peroxidase conjugate solution (Bovine insulin-peroxidase, Sigma, 250 U/mg protein at final concentration of 5 mU/ml in phosphate buffer 40 mM pH 6.8, BSA 0.25%), which was prepared at least 1 h before the experiment and stored in dark at 25 °C, was added and incubated for 2 h in the dark at 25 °C.

Following the incubation, the samples were withdrawn, washed once with 250 µl of cold tap water and dried.

100 µl of chromogene (ABTS®, 9.1 mM, Boehringer-Mannheim) were added and incubated at room temperature (20–25 °C) for 1 or 2 h. The absorbance at 405 nm was read using the MR700 microplate reader (Dynatech) using 100 µl chromogene as blank. For the determination of rat or human insulin levels, rat insulin (Novo Biolabs) or human insulin (Boehringer-Mannheim) prepared in phosphate buffer 40 mM pH 6.8, BSA 0.25%, were used as the respective standards. Standard curves were run in parallel to experimental points at all times.