CRYOPRESERVATION OF FIRST AND INFECTIVE STAGE LARVAE AND
THE INFECTIVITY TEST BY ANIGICAL INOCULATION OF THE NEMATODES
HAEMONCHUS PLACEI AND COOPERIA PUNCTATA

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MATERIALS AND METHODS

Cryopreservation of first-stage larvae
Nematode eggs were recovered from aliquots of the same
faeces used for coprocultures after centrifugation of fecal
suspensions in a sucrose gradient (MARQUARDT, 1961),
followed by three washing steps of centrifugation in water. By
this method approximately 60-80% of
H. placei
and up to 95%
of
C. punctata
eggs were recovered. The eggs were then
transferred to Petri dishes and incubated at room temperature
overnight. The Petri dishes were rinsed with water, larvae washed
twice by centrifugation in 0,85% (5 min, 400 g) and then
resuspended in saline (GILL & REDWIN, 1995). First-stage
larvae were incubated for 10 min in 10% DMSO in 0,85% saline,
transferred to cryovials (50000 or 100000 L1 per vial) and then
placed in dipolystyren inserts, inside an icebox, which was left in
a -80°C freezer for 48-72 h. After this period the vials were
removed from the inserts and plunged into liquid nitrogen.

Cryopreservation of infective larvae
Infective third-stage larvae were obtained by culture of the
faeces of donor calves monospecifically infected with either
H. placei
or
C. punctata
followed by passage through a
Baermann apparatus. Third-stage larvae were exsheathed by
adding 800 µl of a 1% sodium hipochlorite solution to 50 ml
of larval suspension. After mass exsheathment occurred, at
approximately 25 minutes, larvae were washed twice by
centrifugation in distilled water, suspended in saline or distilled
water and transferred to 2,0 ml cryovials (up to 300 000 L3 per
vial). Then they were plunged directly into liquid nitrogen.

Thawing of L3 was carried out by immersing the vials in water
at 37°C until no ice remained, when the larvae were resuspended
in water or saline. After about 1h survival was assessed by counting
dead (burst or non-motile) and live (motile) larvae in ten 10 ml
aliquots of the suspension. L1 were thawed in water at 37°C or
25°C and washed twice in saline, being then resuspended in water.
Survival was assessed in the same way as for L3.

INTRODUCTION

Cryopreservation is probably one of the most effective
means of preserving important species of nematodes for
research and overcoming the problems caused by serial passage
in donor animals, such as alterations in the althelminthic
resistance or pathogenicity of field strains, affecting the
comparison among different studies using the same strains
(GILL & REDWIN, 1995). Besides that, reduction in the need
to keep the expensive, labo and time-consuming infra-structure
donor animals, in particular for cattle nematodes, is per se a
good reason for improving methods of long-term preservation
of helminths. The most common and easiest method of ruminant
nematode cryopreservation uses exsheathed infective larvae,
suspended in water or physiological saline and immersed in the
gas phase of liquid nitrogen (CAMPBELL & THOMSON, 1973; VAN WYK et al., 1977; COLES et al., 1980). However,
exsheathment usually leads to a loss of viability when larvae are
administered per os, requiring injection of larvae directly
into the infection site. This detrimental side-effect has led
research groups to find alternative ways to preserve helminths,
aiming at different life-cycle stages of the parasites. NOLAN et al. (1988) achieved good levels of survival of Strongyloides
stercoralis first-stage larvae (L1) and the infectivity to dogs,
although relatively low, was satisfactory for the maintenance
of the strain. GILL & REDWIN (1995), roptimize cryopreservation conditions for the L1’s of the sheep nematodes
Haemonchus contortus and Trichostrongylus colubriformis,
reaching survival levels in excess of 70%. Infective larvae
derived from frozen L1’s were as infective as normal unfrozen
third-stage larvae, probably due to the fact that the larval sheath
is not lost in the former larvae, as compared to the latter. No
work has been done on cattle nematode L1 cryopreservation,
so we aimed at performing preliminary studies of the conditions
that affect the survival of L1’s of two important cattle nematode
species, H. placei and Cooperia punctata, as well as to confirm
the viability of infective larvae cryopreservation.
Infectivity of thawed third stage larvae

Frozen infective larvae of *H. placei* and *C. punctata* were thawed as described above, and after counting live larvae, enough larval suspension was drawn to achieve 50000 motile larvae of each nematode. These larvae were surgically inoculated into the abomasum (*H. placei*) or the duodenum (*C. punctata*) of a naive Holstein calf aged 6 months. Egg counts were performed daily from DAI (Day after infection) 12 onwards, and the donor calf was sacrificed on DAI 43 for the estimation of worm burden counts done on ten 1% aliquots of either abomasal or small intestinal contents.

**RESULTS**

Survival rates of the third-stage larvae of *H. placei* and *C. punctata* are represented in Table 1. Survival did not seem to be affected by use of saline or distilled water in either freezing or thawing phases. Larvae also survived plunging into liquid nitrogen, although precise cooling rates could not be determined. This is in disagreement with previous studies where ruminant nematode larvae survived poorly or not at all to direct immersion in liquid N2 (COLES et al., 1980).

On the other hand, preliminary results of L1 cryopreservation were very poor for both nematode species in the conditions tested. Thawing conditions were tested, but did not significantly improve survival. Other variables, like cryoprotectant concentration and incubation times, and freezing speed in the first step were not tested, so larvae may have died in the pre-freezing period. Lack of nematode positive faeces prevented testing of these conditions.

The donor calf, injected with both *H. placei* and *C. punctata* thawed infective larvae, began shedding eggs on DAI 15, indicating that *C. punctata* had established (Fig. 1). Egg counts remained high from DAI 18 to 21, declining until DAI 29. Then, on DAI 30, egg counts increased abruptly, reaching a peak of almost 18,000 epg on DAI 38, suggesting an active *H. placei* infection. These results were confirmed by coprocultures from the early and late stages of the experiment, which were composed exclusively of *C. punctata* until DAI 27, with increasing proportions of *H. placei* larvae thereafter, which made up 99% of larvae on DAI 41.

**DISCUSSION**

Cattle nematode infective larvae can be cryopreserved in large numbers for long periods in a rather simple one-step procedure, without any cryoprotectant. We also observed that *H. placei* and *C. punctata* infective larvae survive plunging into liquid N2, as opposite to other studies in which better survival was achieved by the use of a two-step slow freezing technique (COLES et al., 1980). Maybe vitrification occurred, preventing the deleterious formation of intracellular ice that is expected in those larvae, which where not subjected to a “shrinking” step prior to immersion in liquid N2. JAMES (1985) suggested that domestic animal nematode infective larvae that were exposed to freezing temperatures survived better to slow cooling rates probably by synthesis of natural cryoprotectants, what is not the case in Brazil, so slow cooling for infective larvae of tropical regions may not work as wellas for those originating from temperate regions. Use of physiological saline or distilled

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Table 1. Survival of *H. placei* and *Cooperia punctata* exsheathed infective larvae (L3) after freezing in liquid nitrogen.

<table>
<thead>
<tr>
<th>Helminth</th>
<th>Larvae per vial</th>
<th>Freezing medium</th>
<th>Resuspension Medium</th>
<th>% Survival</th>
<th>OBS.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. placei</em></td>
<td>275000</td>
<td>0.9% saline</td>
<td>0.9% saline</td>
<td>91.7</td>
<td>3 months frozen</td>
</tr>
<tr>
<td><em>H. placei</em></td>
<td>300000</td>
<td>0.9% saline</td>
<td>distilled water</td>
<td>74.4</td>
<td>6 months frozen</td>
</tr>
<tr>
<td><em>C. punctata</em></td>
<td>140000</td>
<td>0.9% saline</td>
<td>0.9% saline</td>
<td>82.4</td>
<td>45 days frozen</td>
</tr>
<tr>
<td><em>C. punctata</em></td>
<td>140000</td>
<td>distilled water</td>
<td>0.9% saline</td>
<td>79.2</td>
<td>45 days frozen</td>
</tr>
<tr>
<td><em>C. punctata</em></td>
<td>140000</td>
<td>distilled water</td>
<td>distilled water</td>
<td>75.0</td>
<td>4.5 months frozen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Helminth</th>
<th>Period frozen</th>
<th>Survival</th>
<th>% development to infective larvae*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. placei</em></td>
<td>8-67 days</td>
<td>22.9-27.4 %</td>
<td>2.0</td>
</tr>
<tr>
<td><em>C. punctata</em></td>
<td>6-7 days</td>
<td>24.4-25.8 %</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*aCalculated from the initial number of larvae frozen

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Fig. 1. Egg counts of the donor calf injected in the abomasum with thawed *H. placei* or in the duodenum with thawed *C. punctata* infective larvae.
water for the suspension of larvae in the freezing and/or thawing phases did not change significantly the survival levels, which were high, although they tended to be a little lower in the larvae frozen and/or thawed in distilled water.

First-stage larvae had very low survival levels with the protocol used. When thawing procedures changed, survival did not improve significantly, except in one case (data not shown), so most probably larvae might have died in the freezing or pre-freezing period. Successful cryopreservation of nematode first-stage larvae involved a single slow cooling phase to -70 or -80°C (LOK et al., 1983; GILL & REDWIN, 1995) or two incubation phases in different temperatures and cryoprotectant concentrations (HAM et al., 1981), prior to immersion in liquid nitrogen, or the use of more than one cryoprotectant (NOLAN et al., 1988). All these procedures try to overcome the deleterious side-effects of excessive cell shrinking caused by slow cooling and the intracellular ice formation in rapid cooling, as well as to avoid the toxic effects of prolonged exposure to cryoprotectants (JAMES, 1985). Further studies are being carried out to optimize freezing conditions for L1’s and to test the infectivity of cryopreserved infective larvae to cattle.

ACKNOWLEDGEMENTS

To Dr. Luís Cláudio Lopes C. da Silva of the Veterinary Hospital of the FMVZ-USP for the help in the surgical inoculation of the larvae.

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