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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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 of 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.

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 L_1 per vial) and then placed in dpolystyren 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 L_3 per vial). Then they were plunged directly into liquid nitrogen.

Thawing of L_3 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. L_1 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 L_3.

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

Helminth	Larvae per vial	Freezing medium	Resuspension Medium	% Survival	OBS.
H. placei/L_3	275000	0,9% saline	0,9% saline	91,7	3 months frozen
H. placei/L_3	300000	0,9% saline	distilled water	74,4	6 months frozen
C. punctata/L_3	140000	0,9% saline	0,9% saline	82,4	45 days frozen
C. punctata/L_3	140000	distilled water	0,9% saline	79,2	45 days frozen
C. punctata/L_3	140000	distilled water	distilled water	75,0	4,5 months frozen

Table 2. Survival of *H. placei* and *Cooperia punctata* first-stage larvae after freezing in liquid nitrogen.

Helminth	Period frozen	Survival	% development to infective larvae[a]
H. placei	8-67 days	22,9-27,4 %	2,0
C. punctata	6-7 days	24,4-25,8 %	1,4

[a]Calculated from the initial number of larvae frozen

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 N_2 (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 N_2, 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

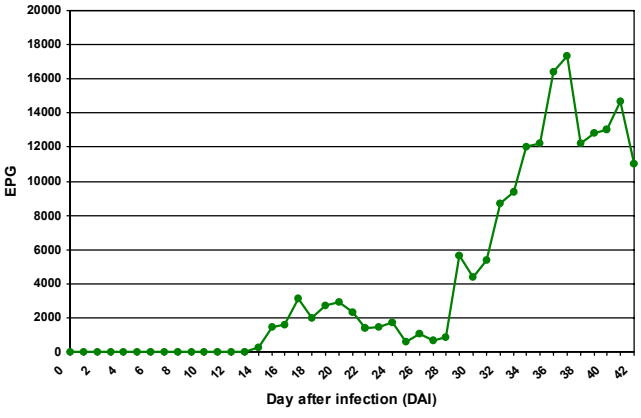

Fig.1. Egg counts of the donor calf injected in the abomasum with thawed *H. placei* or in the duodenum with thawed *C. punctata* infective larvae.

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.

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