Onderstepoort J. vet. Res., 57, 1–6 (1990)

CRYOPRESERVATION OF SOME COMMON NEMATODES OF RUMINANTS FOR UP TO 11,3 YEARS

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ABSTRACT

VAN WYK, J. A., GERBER, H. M. & ALVES, REGINA M. R., 1990. Cryopreservation of some common nematodes of ruminants for up to 11,3 years. *Onderstepoort Journal of Veterinary Research*, 57, 1–6 (1990)

Infective larvae of selected batched of the following nematode species from sheep and cattle were examined for survival and infectivity (by injection into either the abomasum, the duodenum, or the jugular vein) after having been stored in liquid nitrogen for 103–136 months: *Haemonchus contortus, Haemonchus placei, Ostertagia circumcincta, Ostertagia ostertagi, Marshallagia marshalli, Cooperia* spp., *Trichostrongylus axei, Trichostrongylus colubriformis, Trichostrongylus falculatus, Nematodirus spathiger, Nematodirus helvetianus, Oesophagostomum columbianum, Oesophagostomum radiatum, Chabertia ovina* and *Dictyocaulus filaria.*

Excluding *D. filaria*, a mean of 97,7 % of the ovine and 96,0 % of the bovine nematode larvae were alive when thawed.

Compared with previous investigations in this series, little or no reduction occurred with time in either the survival or the viability of the nematodes from cattle, or in the survival of those from sheep. In contrast, the larvae developed poorly in sheep, possibly owing to parenteral treatment of the animals with ivermectin at a dosage of 0,4 mg kg^{-1}, either 6 or 8 days before they were infected.

INTRODUCTION

After Campbell, Blair & Egerton (1972) had shown that the exsheathing of nematode infective larvae (L3) increased their chances of survival when frozen in liquid nitrogen, most of the common species of sheep and cattle and some of dogs and other animals were successfully frozen and thawed without losing their infectivity (Campbell & Thomson, 1973; Campbell Blair & Egerton, 1973; Kelly, Campbell & Whitlock, 1976; Van Wyk, Gerber & Van Aardt, 1977). Notable exceptions were *Gaigeria pachyscelis, Bunostomum phlebotomum* and *Strongyloides papillosus,* which survived freezing and thawing but were not infective thereafter (Van Wyk *et al.*, 1977). Subsequently Van Wyk & Gerber (1980) showed that some worm species could survive and remain infective for up to 59 months of cryopreservation.

While the maximum period of survival of cryopreserved nematode infective larvae in liquid nitrogen reported to date is apparently just short of 5 years (Van Wyk & Gerber, 1980), there is as yet little or no indication of the limits of survival that can be expected.

Some of the batches of L3 of sheep and cattle nematodes used in the initial investigations of Van Wyk *et al.* (1977) and Van Wyk & Gerber (1980) were still available in 1985 after being stored in liquid nitrogen for periods of up to 11,3 years, and it was decided to retest their survival and development.

MATERIAL AND METHODS

Experimental animals

The animals used in the experiments were born and raised under conditions of minimal exposure to worms, on concrete-floored pens swept twice per week for the removal of accumulating manure. Prior to the commencement of the trial, faecal worm egg (epg) counts were negative, both by a modified McMaster method (Reinecke, 1973) and by total flotation of 5 g of faeces (Whitlock, 1959). Nevertheless, as a further precaution, the sheep were dosed subcutaneously with ivermectin[1] (0,4 mg kg^{-1}) either 6 or 8 days before infection, and the calves orally with fenbendazole[2] (10 mg kg^{-1}) 6 days before being infected.

Infective larvae

For our series of investigations (the present investigation, and those by Van Wyk *et al.*, 1977 and by Van Wyk & Gerber, 1980) we selected batches of L3 that were shown to have survived freezing and thawing successfully. The *Cooperia* spp. consist of a mixture of *Cooperia pectinata* and *Cooperia punctata.*

The isolation of the strains of nematodes used in these investigations, as well as their collection, exsheathing, freezing and thawing were described by Van Wyk *et al.* (1977) and Van Wyk & Gerber (1980). In brief, L3 were exsheated in sodium hypochlorite/0,09 % NaC1 solution before being washed with 0,09 % saline and frozen in the gas phase of liquid nitrogen. Thawing occurred in water at 50–55 °C, after which the L3 were diluted with 0,09 % saline.

TABLE 1 Lambs: Route of administration of L3

Group	Sheep	Route of infection		
		Abomasum	Duodenum	Intravenous
A	1–5	*O. circumcincta*	*T. falculatus* *N. spathiger* *C. ovina*	*D. filaria*
B	6–10	*H. contortus* *T. axei* *M. marshalli*	*T. colubriformis* *O. columbianum*	

TABLE 2 Calves: Route of administration of L3

Calf	Route of infection	
	Abomasum	Duodenum
1–3	*H. placei* *O. ostertagi*	*Cooperia* spp. *N. helvetianus* *O. radiatum*

[1] Ivomec (MSD)

[2] Panacur (Hoechst)

Received 31 October 1989—Editor

TABLE 3 Ovine nematodes: percentage of survival and the numbers dosed per sheep

Worm species	Stored larvae		Survival		No. L3 (alive) dosed per sheep
	Concentration*	**Months frozen**	No. examined	**Alive (%)**	
H. contortus	38	**136**	124	**99,2**	3 160
O. circumcincta (a)	12	**136**	65	**100,0**	340
O. circumcincta (b)	44	**136**	331	**97,0**	1 450
M. marshalli (a)	1	**121**	247	**96,8**	5 640
M. marshalli (b)	20	**110**	159	**90,6**	6 310
T. axei	130	**136**	216	**99,1**	11 300
T. falculatus	25	**107**	447	**93,5**	12 310
T. colubriformis	30	**111**	198	**98,0**	9 170
N. spathiger (a)	10	**136**	138	**99,3**	4 320
N. spathiger (b)	25	**115**	146	**99,3**	6 600
O. columbianum	240	**136**	285	**97,9**	6 980
C. ovina (a)	8	**129**	135	**100,0**	3 400
C. ovina (b)	2	**130**	211	**99,1**	3 050
D. filaria (a)	5	**108**	139	**59,0**	4 160
D. filaria (b)	8	**103**	273	**46,9**	2 390
			Mean: †	**97,7 %**	

* Approximate concentration in thousands per mℓ saline during storage

† Arithmetic mean, excluding *D. filaria*

TABLE 4 Bovine nematodes: percentage of survival and the numbers dosed per calf

Worm species	Stored larvae		Survival		No. L3 (alive) dosed per calf
	Concentration*	**Months frozen**	No. examined	**Alive (%)**	
H. placei (a)	16	**134**	199	**97,0**	11 930
H. placei (b)	24	**108**	255	**95,3**	14 920
O. ostertagi (a)	6	**133**	183	**100,0**	3 110
O. ostertagi (b)	18	**108**	200	**99,5**	11 860
Cooperia spp. (a)	14	**118**	193	**86,5**	8 350
Cooperia spp. (b)	230	**118**	200	**91,5**	12 810
N. helvetianus	17	**106**	200	**99,0**	4 510
O. radiatum	8	**136**	155	**99,4**	2 610
			Mean: †	**96,0 %**	

* Concentration in thousands per mℓ saline during storage

† Arithmetic mean

TABLE 5 Number of worms recovered from sheep infected with L3 frozen for 121–136 months

Worm species	Worm stage	Sheep 6
H. contortus	Adult	30
	Development (%)	**1,0 %**
M. marshalli	L4	18
	Adult	341
	Total	359
	Development (%)	**6,4 %**
T. axei	L4	65
	Development (%)	**0,6 %**
T. colubriformis	Adult	3 380
	Development (%)	**36,8 %**
O. columbianum	L3	2
	L4	28
	Adult	4
	Total	34
	Development (%)	**0,5 %**

Percentages of survival of L3 were determined by pipetting the various larval suspensions onto glass slides, and, to stimulate motility, adding formalin to a final concentration of between about 1 % and 3 %. The samples were then examined under a compound microscope. Only larvae that were seen to move were counted as alive, a procedure that considerably slowed down the examination, as many of the L3 (and even some entire batches) were lethargic. This necessitated long periods of observation before individual L3 could be counted as dead. Larvae that had burst were disregarded when the survival of L3 were estimated, because, although not thoroughly tested, previous investigations had indicated that their proportions in a given batch of L3 appeared to be constant, irrespective of the period of cryopreservation (Van Wyk *et al.*, 1977). The percentages of survival were determined after the L3 had been thawed for at least 4 h, but less than eight.

Sufficient L3 of *Haemonchus contortus, Trichostrongylus axei, Trichostrongylus falculatus, Trichostrongylus colubriformis, Oesophagostomum columbianum* and *Oesophagostomum radiatum* remained from the batches used in the previous investigations by Van Wyk *et al.* (1977) and Van Wyk & Gerber (1980) for all of the animals used in the present trial to be infected therewith. However, in the case of *Marshallagia marshalli, Ostertagia circumcincta, Nematodirus spathiger, Chabertia ovina, Dictyocaulus filaria, Cooperia* spp., *Nematodirus helvetianus, Haemonchus placei* and *Ostertagia ostertagi,* as there were insufficient L3 of the batches used in the previous investigation, other batches had to be used for infecting either some or all of the animals used in the present investigations.

Infection of the animals

The sheep in Group A (Table 1) were infected on 1985/03/25, those in Group B on 1985/03/27, and all 3 calves (Table 2) on 1985/05/07. On each of the 3 days the L3 were thawed commencing 04:00, and while the sheep were infected during the course of the morning, approximately between 09:00 and 12:00, the calves were infected only at 13:45–15:15 in the afternoon.

All the L3 were administered by injection with a

TABLE 6 Number of worms recovered from calves infected with L3 frozen for 106–136 months

Worm species	Worm stage	Calf			
		1	2	3	Mean for group
H. placei (a)	Adult	352	–	–	352
	Development (%):	**3,0**	–	–	**3,0%**
H. placei (b)	Adult	–	1 016	326	671
	Development (%):	–	**6,8**	**2,2**	**4,5%**
O. ostertagi (a)	Adult	696	–	–	696
	Development (%):	**22,4**	–	–	**22,4%**
O. ostertagi (b)	Adult	–	1 813	685	1 249
	Development (%):	–	**15,3**	**5,8**	**10,6%**
Cooperia spp. (a)	L4	73	–	–	73
	Adult	190	–	–	190
	Total	263	–	–	263
	Development (%):	**3,2**	–	–	**3,2%**
Cooperia spp. (b)	L4	–	30	118	74
	Adult	–	41	2 437	1 239
	Total	–	71	2 555	1 313
	Development (%):	–	**0,6**	**19,9**	**10,3%**
N. helvetianus	L4	0	0	3	1
	Adult	29	342	3 215	1 195
	Total	29	342	3 218	1 196
	Development (%):	**0,6**	**7,6**	**71,4**	**26,5%**
O. radiatum	L3	33	43	0	25
	L4	45	23	74	47
	Adult	519	365	846	577
	Total	597	431	920	649
	Development (%):	**22,9**	**16,5**	**35,3**	**24,9%**

TABLE 7 Ovine nematodes: Comparison of the survival of L3 cryopreserved for various periods of time

Worm species†	Survival (%)*			
	After +/– 7,5 months	After +/– 23 months	After +/– 59 months	After 103–136 months
H. contortus	85,9	96,6	97,2	99,2
O. circumcincta (a)	96,6	95,6	77,2	100,0
O. circumcincta (b)	–	–	–	97,0
M. marshalli (a)	–	–	86,3	96,8
M. marshalli (b)	–	–	–	90,6
T. axei	98,0	96,7	97,6	99,1
T. falculatus	–	–	94,1	93,5
T. colubriformis	100,0	93,5	95,9	98,0
N. spathiger (a)	97,0	91,0	95,2	99,3
N. spathiger (b)	–	–	–	99,3
O. columbianum	83,3	87,3	88,9	97,9
C. ovina (a)**	87,9	86,8	100,0	100,0
C. ovina (b)	–	–	–	99,1
D. filaria (a)	–	–	49,3	59,0
D. filaria (b)	–	–	–	46,9
Mean survival (excluding *D. filaria*)	**92,7 %**	**92,5 %**	**92,5 %**	**97,7 %**

* See van Wyk *et al.* (1977), Table 12; Van Wyk & Gerber (1980), Table 1; and Table 3 above for the exact periods of storage

† All the data in a given row pertain to a single batch of larvae frozen on the same day

** *C. ovina* was frozen for approximately 7 months, and *T. falculatus* for approximately 29 months shorter than most of the others

hypodermic syringe, via the routes listed in Tables 1 and 2. With the exception of the *D. filaria* L3, which were injected intravenously into the jugular vein, the L3 were deposited in either the abomasum or the duodenum of each animal by means of laparotomy operations, conducted under sedation with xylacine HC1[3] and local anaesthesia with procaine HC1[4]. Immediately after the operation, each sheep was treated intramuscularly with 3 g of long-acting penicillin[5].

Worm recovery and identification

The abomasal, small intestinal and large intestinal ingesta of the trial animals were concentrated by sequential sieving through sieves having apertures of 150 µm and 37 µm. The residues on both sieves were retained for worm recovery.

The total worm burdens listed in the results are the sum of microscopic examination of 2 × 1/10 aliquots of the ingesta of the various gastrointestinal organs and total microscopic examination of their digested mucosa.

The first 25 worms in each sample were mounted and identified, but in those samples that contained fewer than 25 worms all were identified.

RESULTS

Particulars of the ovine and bovine L3 that were frozen and thawed, their survival after cryopreservation for 103–136 months and the numbers that were administered to the trial animals are listed in Tables 3 and 4, respectively.

If the results of the 2 batches of *D. filaria* (mean of 53 % survival) are excluded, a mean of 97,7 % (range 90,6–100 %) of 9 species of ovine and 96 % (range 86,5–100 %) of 5 species of bovine nematodes survived the periods of cryopreservation of up to 11,3 years (Tables 3 and 4). The ranges for those

[3] Rompun (Bayer)

[4] Planocaine (Maybaker)

[5] Compropen (Milborrow)

TABLE 8 Bovine nematodes: Comparison of the survival of L3 cryopreserved for various periods of time

Worm species†	Survival (%)*			
	After 1–5 months	After 24–28 months	After 55–59 months	After 103–136 months
H. placei (a)	43,8	86,2	86,7	97,0
H. placei (b)	—	—	—	95,3
O. ostertagi (a)	90,0	96,3	96,6	100,0
O. ostertagi (b)	—	—	—	99,5
Cooperia spp. (c)	89,2	97,2	97,1	—
Cooperia spp. (a)	—	—	95,1	86,5
Cooperia spp. (b)	—	—	—	91,5
N. helvetianus (a)	99,2	98,6	97,2	—
N. helvetianus (b)	—	—	97,6	91,5
O. radiatum	90,6	95,1	95,1	99,4
Mean survival	**82,6 %**	**94,7 %**	**95,1 %**	**95,1 %**

* See Van Wyk *et al.* (1977), Table 20; Van Wyk & Gerber (1980), Table 2; and Table 4 above for the exact periods of storage. The *Cooperia* spp. (a) listed in the column, "55–59 months", had been stored for only 39 months

† All the data in a given row pertain to a single batch of larvae frozen on the same day

TABLE 9 Ovine nematodes: Comparison of the infectivity of L3 cryopreserved for various periods of time

Worm species†	Development (%)*			
	After +/– 7,5 months	After +/– 23 months	After +/– 59 months	After 103–136 months
H. contortus	31,7	39,9	35,7	1,0
O. circumcincta (a)	18,6	57,5	40,2	—
M. marshalli (a)	—	—	6,2	—
M. marshalli (b)	—	—	—	6,4
T. axei	9,4	27,7	22,9	0,5
T. colubriformis	45,5	62,7	37,7	36,8
N. spathiger (a)	27,6	63,8	25,2	—
O. columbianum	18,1	24,9	3,8	0,5
*C. ovina***	—	38,8	24,0	—
Mean development	**25,2 %**	**45,0 %**	**24,5 %**	—

* See van Wyk *et al.* (1977), Table 12; Van Wyk & Gerber (1980), Table 1; and Table 3 above for the exact periods of storage. For the first 3 periods infection *per os* and by intravenous injection are excluded. The last period shows development in 1/10 sheep only, in the wake of treatment with ivermectin

† All the data in a given row pertain to a single batch of larvae frozen on the same day

** *C. ovina* was frozen for approximately 7 months, and *T. falculatus* for approximately 29 months shorter than most of the others

L3 that were stored for more than 11 years were 97,0–100 % for both the ovine and the bovine nematodes. *Cooperia* spp. (a) and *Cooperia* spp. (b), both of which originated from the same batch of L3 frozen on the same day and which differed only in the concentration of L3 during storage, 86,5 and 91,5 %, respectively, of the L3 survived.

Worm recoveries from the various animals are shown in Tables 5 and 6. Worms were recovered from only 1 of the 10 sheep infected in the trial, with percentages of development ranging from 0,6 % (*T. axei*) to 36,8 % (*T. colubriformis*). In contrast, worms developed in each of the 3 calves and the mean percentages of development in these ranged from 3,0 % (one of 2 batches of *H. placei*) to 26,5 % (*N. helvetianus*). The development of *Cooperia* spp. (a) in the single calf in which it was tested was 3,2 %, and that of *Cooperia* spp. (b) in 2 calves, 0,6 % and 19,9 %.

In Tables 7 and 8 comparisons are drawn for sheep and cattle, respectively, between the survival rates of the various nematode species and batches of larvae cryopreserved for 103–136 months (results of the present investigations), and the results of 3 previous trials, in which L3 were thawed after approximately 8 months, 23 months and 59 months of cryopreservation. In the case of the nematodes (excluding *D. filaria*) from sheep, the mean survival per trial varied from 92,5–97,7 % (Table 7) and, for those of cattle, from 82,6 % (after 1–5 months of cryopreservation) to 95,1 % (after both 55–59 months and 103–136 months; Table 8).

In Tables 9 and 10, similar comparisons are drawn for the development of the thawed L3 in the 2 host species. The ranges of mean development were 24,5–45,0 % in the nematodes from sheep (Table 9), but this excludes the results of the present trial, in which only 1 sheep became infected. The corresponding ranges in all 4 investigations in the nematodes from cattle were 12,2 % (after 1–5 months) to 21,2 % (after 55–59 months; Table 10).

In Table 11, the worm larvae (the sum of parasitic 3rd and 4th stages), recovered at necropsy, are expressed as percentages of the total worm burdens of the calves. The larvae constituted from 0 % (*H. placei* and *O. ostertagi*) to 11,2 % (*O. radiatum*) of the total.

DISCUSSION

Van Wyk *et al.* (1977) stated that Weinman & McAllister (1947) were apparently the first to report on the survival of nematode larvae after freezing and thawing in the laboratory. However, since then it has came to our notice that this had already been demonstrated at least 15 years previously, as Ober-Blöbaum (1932) successfully cryopreserved the L3 of equine strongyles, amongst others by air-drying L3 on slides and then freezing them.

TABLE 10 Bovine nematodes: Comparative development of L3 cryopreserved for various periods of time

Worm species†	Survival (%)*			
	After 1–5 months	After 24–28 months	After 55–59 months	After 103–136 months
H. placei (a)	14,5	10,6	15,4	3,0
H. placei (b)	—	—	—	4,5
O. ostertagi (a)	19,8	36,1	38,6	22,4
O. ostertagi (b)	—	—	—	10,6
Cooperia spp. (c)	23,1	0,4	8,9	—
Cooperia spp. (a)	—	—	0,0	3,2
Cooperia spp. (b)	—	—	—	10,3
N. helvetianus (a)	3,5	12,0	12,6	—
N. helvetianus (b)	—	—	50,7	26,5
O. radiatum	0,2	5,0	22,5	24,9
Mean development	**12,2 %**	**12,8 %**	**21,2 %**	**13,2 %**

* See Van Wyk *et al.* (1977), Table 20; Van Wyk & Gerber (1980), Table 2; and Table 4 above for the exact periods of storage. The *Cooperia* spp. (a) listed in the column, "55–59 months", had been stored for only 39 months

† All the data in a given row pertain to a single batch of larvae frozen on the same day

TABLE 11 Larvae expressed as a percentage of the total number of worms recoverd

Worm species	Worm recovery	
	Total number	Larvae (%)
H. placei	1 694	0
O. ostertagi	3 194	0
Cooperia spp.	2 889	7,6
N. helvetianus	3 589	0,1
O. radiatum	1 948	11,2

It must be kept in mind that, although not specifically mentioned previously, selected batches of cryopreserved L3 were used in these, as in the other investigations by the present authors (Van Wyk *et al.*, 1977; Van Wyk & Gerber, 1980). The importance of this fact is evident from the results of Kelly & Campbell (1974), who reported such variations in the survival and infectivity of cryopreserved L3 both between different batches and between different vials from the same batch, that they expressed reservations about the technique as a standard laboratory procedure. Thus, the results of our series of experiments should certainly not be interpreted as though such good results are to be expected with all the vials in all batches of cryopreserved L3 of these common worm species.

Statistical comparisons between our various experiments conducted since 1973 are not valid, since some of the batches of L3 differed in the different investigations. Nevertheless, there seems to be no indication that either the survival rates or the infectivity of cattle nematode L3 are declining after up to 11,3 years of storage in the gas phase of liquid nitrogen. However, the results of *O. circumcincta* need to be considered. The L3 thawed after 59 months of cryopreservation showed a drop in the percentage of survival from more than 95 % to 77,2 % (Van Wyk & Gerber, 1980). These authors reported that some of these L3 were flattened in appearance and concluded that, although this could have been an indication of aging, it was not possible to exclude the possibility that it was owing to a variation between the different ampoules of L3 in the batch concerned. In the present trial, 100 % of the L3 of *O. circumcincta* (of the same batch as before) that were examined when the percentage of survival was estimated, were alive (Table 7), and did not appear flattened. Thus, although there seems to be no explanation for the phenomenon and the thawing process cannot be excluded as a possible contributory factor, it seems probable that the relatively poor results after 59 months of storage compared to those in the previous and subsequent examinations, were due to variations between different vials of this batch of L3.

This appears to be the first report of nematode larvae having survived cryopreservation for up to 11,3 years without losing their infectivity. Admittedly, the development was poor in sheep, as only 1 out of 10 animals developed any infection at all, and even in that sheep only *T. colubriformis* developed satisfactorily. However, these sheep had been treated parenterally with ivermectin at 0,4 mg kg^{-1} (twice the therapeutic dosage registered in South Africa) either 6 or 8 days before they were infected, and Armour, Bairden, Batty, Davidson & Ross (1985) showed that this compound had some residual activity in cattle treated by this route. The apparently unchanged infectivity of the nematodes of cattle after the extended period of storage (13,2 % in the present experiment, compared with means of 12,2 %, 12,8 % and 21,2 % in the first 3 trials—Table 10), indicate that development should be retested in sheep before it can be concluded that there has been a reduction in the viability of the ovine nematode L3.

A question that arose from previous work is whether the concentration of the stored L3 is important, and whether there is a maximum concentration which should not be exceeded. When we started investigating cryopreservation in liquid nitrogen as a possible standard technique in the laboratory, we froze different concentrations of some batches of L3 of a given species on the same day. The 2 different concentrations of *Cooperia* spp. used in the present trial, constitute such an example. Both survived well (Table 4), and each has been tested in 2 calves, with nil development in the 1st calf (Van Wyk & Gerber, 1980, Table 9, Calf 2) in which the lower concentration was tested, and 3,2 % in the 2nd (Table 6 above), compared with 0,6 % and 19,9 % development of the L3 of the higher concentration (Table 6 above).

Obviously, these few tests cannot be regarded as sufficient for a thorough testing of the null hypothesis as regards a possible effect of concentration on the survival and viability of L3. Both concentrations of *Cooperia* spp. showed much variability, and there is at present no apparent reason for rejecting the null hypothesis, particularly since 2 other worm species gave relatively good results despite concentrations of 130 000 and 240 000 L3 of *T. axei* and *O. colum-*

bianum, respectively (Van Wyk *et al.*, 1977; Van Wyk & Gerber, 1980; and Tables 3 and 5 above). Thus it would appear that, if there is a critical concentration of L3 that should not be exceeded, it is probably higher than a quarter of a million L3 per mℓ saline. On the other hand, it is not valid to extrapolate to species other than those that have been tested.

The present trials did not include work on the nematodes, *G. pachyscelis*, *B. phlebotomum* and *S. papillosus*, that infect sheep and cattle percutaneously. This should receive further attention, seen in the light of the fact that, by using cryoprotectants and controlled rates of freezing, Nolan, Aikens & Schad (1988) did manage successful cryopreservation of *Strongyloides stercoralis* with retention of infectivity for dogs.

Larvae constituted only very small proportions (0–11,3 %) of the namatodes recovered from the calves used in these experiments. Thus, despite the fact that no attempt was made to classify them according to the presence or absence of hypobiosis, it seems reasonable to conclude from their low prevalence that the prolonged periods of storage in liquid nitrogen did not substantially retard development. This is in agreement with the results of Van Wyk *et al.* (1977), and is supported by those of Hendrikx, Boersema & Eysker (1988), who concluded that cryopreservation may have affected the level of hypobiosis in their investigations. They had recovered considerably lower proportions of early fourth-stage larvae (of a strain of *H. contortus* selected for hypobiosis) from sheep infected with cryopreserved, than in others infected with unfrozen larvae.

Van Wyk *et al.* (1977) concluded that the costs involved in laparotomies made the use of this technique of cryopreservation impractical unless L3 remained viable for at least 2 years. The present results certainly dispel any doubts that may have remained on that count. In fact, it can be concluded that this technique is becoming almost indispensible for inexpensive and effective maintenance of nematode strains in the laboratory.

ACKNOWLEDGEMENTS

The authors are indebted to Dr C. C. Kingsley for undertaking the laparotomy operations required for infecting the experimental animals.

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Printed by the Government Printer, Private Bag X85, Pretoria, 0001