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SURVIVAL AND DEVELOPMENT OF LARVAE OF THE COMMON NEMATODES OF RUMINANTS AFTER LONG-TERM CRYOPRESERVATION AND INVESTIGATION OF DIFFERENT ROUTES OF INFESTATION

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ABSTRACT

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Exsheathed infective larvae (L3) of 16 species of nematodes were tested for infectivity in either sheep or cattle after they had been frozen and stored in 0,09% NaCl solution in the gas phase of liquid nitrogen for periods of up to 59 months.

A mean of >90% of the L3 of *Haemonchus contortus*, *Ostertagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Nematodirus spathiger*, *Oesophagostomum columbianum* and *Chabertia ovina* of sheep and *Haemonchus placei*, *Ostertagia ostertagi*, *Cooperia* spp. (*C. pectinata* and *C. punctata*), *Nematodirus helvetianus* and *Oesophagostomum radiatum* of cattle was alive when they were thawed, after having been frozen for 52–59 months.

These L3 as well as those of *Marshallagia marshalli* and *Trichostrongylus falculatus*, which had been frozen for 30–33 months, were infective to sheep or cattle when dosed *per os* or inoculated into the abomasum or the duodenum. Thawed *Dictyocaulus filaria* L3, frozen for 31 months, developed poorly when injected intravenously into sheep.

This appears to be the first report showing infectivity of L3 of *O. circumcincta*, *T. colubriformis*, *N. spathiger* and, possibly, of *O. radiatum* by the oral route after cryopreservation in liquid nitrogen.

Résumé

SURVIVANCE ET DÉVELOPPEMENT DES LARVES DE NÉMATODES COMMUNS DES RUMINANTS APRÈS UNE CRYO-CONSERVATION DE LONGUE PÉRIODE ET INVESTIGATION DES DIFFÉRENTES VOIES D'INFESTATION

Des larves infectieuses dépourvues de leur enveloppe (L3) de seize espèces de nématodes ont été testées en ce qui concerne leur infectivité soit chez le mouton, soit chez le bétail après avoir été congelées et conservées dans une solution de NaCl à 0,09% dans la phase gazeuse d'azote liquide pendant des périodes allant jusqu'à 59 mois.

Une moyenne de >90% de L3 de Haemonchus contortus, Ostertagia circumcincta, Trichostrongylus axei, Trichostrongylus colubriformis, Nematodirus spathiger, Oesophagostomum columbianum *et* Chabertia ovina *du mouton et* Haemonchus placei, Ostertagia ostertagi, Cooperia *spp.* (C. pectinata *et* C. punctata), Nematodirus helvetianus *et* Oesophagostomum radiatum *du bétail étaient vivants quand ils furent dégelés, après avoir été congelés pendant une période de 52–59 mois.*

Ces L3 ainsi que celles de Marshallagia marshalli *et* Trichostrongylus falculatus *qui avaient été congelées pendant* 30–33 *mois, étaient infectieuses pour le mouton ou le bétail quand on less injectait* per os *ou quand elles étaient inoculées dans l'abomasum ou le duodenum. Les* Dictyocaulus filaria *L3, dégelés, congelés pendant* 31 *mois, se développèrent médiocrement quand ils furent injectés de manière intraveineuse au mouton.*

Ceci semble être le premier rapport montrant l'infectivité des L3 de O. circumcincta, T. colubriformis, N. spathiger *et, sans doute,* O. radiatum *par voie orale après cryo-conservation dans l'azote liquide.*

INTRODUCTION

Exsheathed infective larvae (L3) of many nematode species of ruminants, rodents and carnivores survive cryopreservation in the gas phase of liquid nitrogen and retain some infectivity (Campbell, Blair & Egerton, 1972; Campbell & Thomson, 1973; Campbell, Blair & Egerton, 1973; Kelly & Campbell, 1974; Kelly, Campbell & Whitlock, 1976). Some nematodes of ruminants remained infective after cryopreservation for up to 28 months (Van Wyk, Gerber & Van Aardt, 1977). Other studies (Van Wyk & Gerber, 1980) suggest that strain characteristics are unchanged after cryopreservation.

In most of the trials conducted by Van Wyk *et al.* (1977), the infectivity of thawed L3 was tested by injection during laparotomy into either the abomasum or the duodenum. To obviate laparotomy operations and thus reduce costs, these workers suggested trials to test alternative routes of infestation for those species of worms which could be stored successfully.

The trials reported in this paper were planned to study the development of L3 frozen for almost 5 years and to test alternative routes of infestation with cryopreserved L3.

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MATERIALS AND METHODS

Experimental animals

Dorper sheep were used in the investigations and calves of Friesian or Fresian × Jersey origin, all of which, both sheep and calves, were raised worm-free, fed sterilized lucerne hay and kept under worm-free conditions during the experiments. Before infestation their worm-free condition was confirmed by the examination of faecal specimens. As a further precaution the animals were treated with levamisole* at 15 mg/kg live mass at least 3 days before infestation.

Infective material

Infective larvae (L3) of *Haemonchus contortus*, *Ostertagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Nematodirus spathiger*, *Oesophagostomum columbianum*, *Dictyocaulus filaria*, *Chabertia ovina*, *Haemonchus placei*, *Ostertagia ostertagi* and *Oesophagostomum radiatum* left over from previous cryopreservation trials (Van Wyk *et al.*, 1977, Experiments 2, 3, 12 & 13), were used in the present investigations. Insufficient *Nematodirus helvetianus* and *Cooperia* spp. larvae were available for use in all the animals and so larvae stored for a shorter period were used in some of the calves.

* Ripercol (Ethnor)

The isolation of these strains of nematodes in the laboratory and the collection, exsheathing, freezing and thawing of L3 have been described previously (Van Wyk *et al.*, 1977).

Briefly, larvae were exsheathed in freshly prepared 0,15% sodium hypochlorite/physiological saline solution, were washed with saline on filter paper discs in a Buchner funnel, concentrated by sedimentation and sealed in thin-walled glass ampoules before being submerged in the gas phase of liquid nitrogen. The ampoules of frozen larval suspension were thawed in hot water at 50–55 °C until only a small piece of ice remained, transferred to water at room temperature and then diluted with 0,09%† saline solution.

Details of the duration of freezing, viability, and numbers of larvae dosed to the experimental animals are listed in Tables 1 & 2.

The routes of administration of L3 of the various ovine and bovine worm species are listed in Tables 3 & 4, respectively.

Faecal egg counts were carried out on 2 occasions, 19–21 days and 23–25 days after infestation respectively, and the animals were killed 27–30 days after infestation.

TABLE 1 Ovine nematodes: viability of stored larvae and the numbers dosed per sheep

Worm species	Stored larvae		Viability		No. L3 (alive) dosed per sheep	
	Concentration*	**Months frozen**	No. examined	**Alive (%)**	Groups A & B	Group C
H. contortus	38	**59**	251	**97,2**	9 560	9 560
O. circumcincta	12	**59**	180	**77,2**	2 223	2 223
M. marshalli	20	**33**	226	**86,3**	11 996	—
T. axei	130	**59**	211	**97,6**	13 322	13 322
T. falculatus	25	**30**	205	**94,1**	22 114	88 454
T. colubriformis	2	**59**	147	**95,9**	967	—
N. spathiger	10	**59**	187	**95,2**	5 331	5 331
O. columbianum	240	**59**	216	**88,9**	7 468	26 137
C. ovina	8	**52**	125	**100,0**	2 600	2 600
D. filaria	5	**31**	73	**49,3**	—	3 510

* Concentration in thousands per mℓ saline during storage

TABLE 2 Bovine nematodes: viability of stored larvae and the numbers dosed per calf

Worm species	Stored larvae		Viability		No. L3 (alive) dosed per calf			
	Concentration*	**Months frozen**	No. examined	**Alive (%)**	Calf 1	Calf 2	Calf 3	Calf 4
H. placei	16	**56**	173	**86,7**	10 664	10 664	10 664	10 664
O. ostertagi	6	**55**	89	**96,6**	4 347	4 347	4 347	4 347
N. helvetianus (1974)	3	**58**	71	**97,2**	3 927	—	—	—
N. helvetianus (1976)	17	**28**	124	**97,6**	—	18 856	18 856	13 469
Cooperia spp. (1973)	14	**59**	137	**97,1**	12 468	—	12 468	—
Cooperia spp. (1975)	14	**40**	123	**95,1**	—	15 406	—	15 406
O. radiatum	8	**57**	81	**95,1**	8 464	8 464	8 464	8 464

* Concentration in thousands per mℓ saline during storage

TABLE 3 Routes of infestation of sheep

Group	Sheep	Route of infestation			
		Abomasum (injection)	Duodenum (injection)	*Per os*	Intravenous (injection)
A	1–5	*H. contortus* *T. axei* *M. marshalli*	*T. colubriformis* *O. columbianum*	—	—
B	6–10	*O. circumcincta*	*T. falculatus* *N. spathiger* *C. ovina*	*T. axei*	*D. filaria*
C	11–15	—	—	*H. contortus* *O. circumcincta* *T. falculatus* *N. spathiger* *O. columbianum*	—

† N.B. *Erratum.*—The concentration of NaCl is incorrectly reported in Van Wyk *et al.* (1977); for 0,9% read 0,09%

TABLE 4 Routes of infestation of calves

Calves	Route of infestation		
	Abomasum (injection)	Duodenum (injection)	*Per os*
1–2	*H. placei* *O. ostertagi*	*N. helvetianus* *Cooperia* spp. *O. radiatum*	—
3–4	—	—	*H. placei* *O. ostertagi* *N. helvetianus* *Cooperia* spp. *O. radiatum*

Worm recovery

The gastro-intestinal organs were collected at necropsy as described by Reinecke (1973). The worms were recovered from the gastro-intestinal ingesta by migration, using a modified gelled-agar technique (Van Wyk, 1978; Van Wyk, Gerber & Groeneveld, 1980).

Briefly, before being submerged in physiological saline in a water-bath at 40 °C for overnight incubation, the gastro-intestinal ingesta were gelled in agar* (final strength 0,3–0,35% agar) around mesh supported in a perspex frame with a removable base-plate. After the worms migrating from the agar plates had been rinsed and wiped off by hand, the agar gel was melted and, while hot, removed from the ingesta by sieving. The ingesta were retained for the determination of the numbers of worms that had failed to migrate from the agar.

Worm counts and worm identification

Because the samples from this trial were also used to assess the efficacy of the gelled-agar method of worm recovery (Van Wyk *et al.*, 1980), all of them were examined *in toto* and microscopically (stereomicroscope) for worms.

In each sample the first 50 worms recovered (or 150–200 worms in samples containing numerous worm species) were identified, but if fewer than 50 worms (or 150–200) were recovered, all were identified (Reinecke, 1973). Because female *Trichostrongylus* could not be identified to species level, the ratio of males of the different species was determined where more than one species occurred in the same sample, and the females were divided accordingly.

For *N. spathiger* the numbers of ova occurring in worms from sheep of Group B (duodenal infestation) and Group C (*per os* infestation) were determined with the aid of a standard microscope. Student's t-test (Steel & Torrie, 1960) was used to test whether the worm egg counts of the 2 groups differed significantly.

RESULTS

D. filaria L3 were lethargic, as is usual for the species, but those of the other species were very active when thawed. Apart from *D. filaria* L3, whose survival rate was 49,3% (Table 1), the survival of the other L3 of ovine origin varied from 77,2% (*O. circumcincta*, frozen for 59 months) to 100,0% (*C. ovina*, frozen for 52 months). For bovine species (Table 2), the survival rate varied from 86,7% (*H. placei*, frozen for 56 months) to 97,6% (*N. helvetianus* frozen for 28 months).

Many of the dead *O. circumcincta* (22,8%) had the flattened appearance of adult *Schistosoma mattheei* that have been frozen and thawed (Van Wyk, unpublished data, 1971). These dead larvae, however, resembled live L3 in that they were often kinked, whereas dead intact larvae are usually straight or only slightly bent.

Apart from *N. spathiger*, the mean faecal egg counts per gram (e.p.g.) of the sheep were 31 775 and 22 033 on 19–21 and 23–25 days after infestation for Group A, 720 and 920 for Group B and 9 925 and 11 667 for Group C (Table 5) respectively. For the calves the corresponding mean counts were 225 and 1 025 for the 2 calves infested by injection and 275 and 675 for the 2 infested *per os* (Table 6).

TABLE 5 Sheep: faecal egg counts

Group	Sheep	Days after infestation			
		19–21		23–25	
		Other spp.	*N. spatigher*	Other spp.	*N. spathiger*
A	1	33 500	0	21 300	0
	2	5 200	0	—*	—
	3	45 500	0	—	—
	4	—	—	22 800	0
	5	42 900	0	22 000	0
	Mean	**31 775**	**0**	**22 033**	**0**
B	6	700	0	2 200	0
	7	600	400	800	500
	8	500	400	300	200
	9	0	100	100	300
	10	1 800	0	1 200	100
	Mean	**720**	**180**	**920**	**220**
C	11	16 900	0	—	—
	12	3 900	0	1 500	0
	13	8 800	0	—	—
	14	10 100	0	6 600	0
	15	—	—	26 900	0
	Mean	**9 925**	**0**	**11 667**	**0**

* No faecal egg count done

TABLE 6 Calves: faecal egg counts

Method of infestation	Calf	Days after infestation			
		19		23	
		Other spp.	*Nematodirus*	Other spp.	*Nematodirus*
Injection....	1	350	0	1 300	0
	2	100	100	750	700
	Mean	**225**	**50**	**1 025**	**350**
Per os.....	3	550	0	1 300	0
	4	0	0	50	50
	Mean	**275**	**0**	**675**	**25**

* Commercial grade

TABLE 7 Uterine egg counts of *N. spathiger* from Groups B & C

Route of infestation/sheep	Eggs in uterus of *N. spathiger*				Number of worms examined
	Mean	Standard deviation	Minimum	Maximum	
Intraduodenal (Group B)					
6	9,4	7,2	0	27	26
7	17,5	9,5	6	36	24
8	14,9	9,3	0	31	21
9	21,4	10,4	4	42	23
10	17,6	6,4	3	30	20
Mean	**16,0***	**9,5**	—	—	—
Per os (Group C)					
11	7,3	6,2	0	21	21
12	7,3	4,6	0	19	18
14	18,9	8,5	6	32	8
15	17,1	7,1	5	30	19
Mean	**11,5***	**8,1**	—	—	—

* The difference between the 2 groups is significant ($P<0,01$)

No *Nematodirus* eggs were recovered from the faeces of Groups A and C of the sheep, but means of 180 and 220 e.p.g. were recovered in Group B 19–21 and 23–25 days, respectively, after infestation. *Nematodirus* ova were recovered from both groups of calves, the means being 50 and 0 e.p.g. at 19 days and 350 and 25 e.p.g. at 23 days after infestation.

The mean intra-uterine egg count (Table 7) of 114 *N. spathiger* from Group B (intraduodenal infestation) was 16,0 (±9,5; range 0–42) and that of 66 *N. spathiger* from Group C (*per os* infestation) was 11,5 (±8,1; range 0–32). The difference between the 2 groups was significant ($P<0,01$).

The mean development of sheep nematodes varied from 6,2% (*M. marshalli*) to 40,2% (*O. circumcincta*) for infestations by intra-abomasal injection, from 2,7% (*T. falculatus*) to 37,7% (*T. colubriformis*) for infestations by intra-duodenal injection and from 0,2% (*T. falculatus*) to 16,6% (*H. contortus*) for *per os* infestations (Table 8). A mean of 0,6% *D. filaria* (injected intravenously) developed. The corresponding variations for calves were 4,0% (*Cooperia* spp.) to 38,6% (*O. ostertagi*) for infestation by duodenal or abomasal injection and 0,02% (*O. radiatum*) to 4,3% (*O. ostertagi*) for *per os* infestations (Table 9). Unfortunately, insufficient numbers of L3 of *Cooperia* spp. and of *N. helvetianus* of the original batches necessitated the use of larvae from more than 1 batch in this trial (Table 2).

The survival and development of the same batches of 7 of the ovine and 5 of the bovine nematode species had been tested previously after shorter periods of storage in the gas phase of liquid nitrogen (Van Wyk *et al.*, 1977). Tables 10–13 were compiled to compare the survival and development of the L3 in the 2 trials to ascertain whether the prolonged storage had a deleterious effect on the viability of the larvae.

Discussion

Survival of L3

Despite the prediction that nematode L3 may survive cryopreservation for "1–2 years or longer" (Weinman & McAllister, 1947), it was amazing that such large percentages of the L3 were alive when thawed after having been frozen for almost 5 years (Tables 1 & 2). Indeed, comparisons of survival of the same batches of larvae (Tables 10 & 11), show that only in the case of *O. circumcincta* did the survival rate of L3 drop more than 1,5% between 2 and 5 years of cryopreservation, while slightly higher percentages of survival were recorded after 5 years than after 2 years for *H. contortus, T. colubriformis, N. spathiger, O. columbianum* and *C. ovina* of sheep and for *H. placei* and *O. ostertagi* of cattle. This was undoubtedly due to variations in the viability of different ampoules of larvae.

It is interesting that some L3 *O. circumcincta,* the only species with relatively poor survival after 5 years of cryopreservation, were flattened in appearance. This phenomenon was not observed when L3 of the same batch were thawed after shorter periods of storage and it may therefore be a sign of their ageing. On the other hand, the possibility that this was due to a variation between the different ampoules of L3 in this batch cannot be excluded.

Faecal egg counts

The high faecal egg counts in Groups A and C of the sheep and the high counts in Group B (sheep) and in calves 1–3 seem to confirm the supposition of Van Wyk *et al.* (1977) that the egg-laying capacity of the worms was not affected by prolonged freezing of the L3. It is surprising, however, that no *Nematodirus* ova were detected in the lambs of Group C, despite the fact that they had a mean burden of 352 5th stage and adult *N. spathiger*, many of which contained ova. By contrast numerous *Nematodirus* ova were detected in the faeces of Group B with a mean of 1 310 worms.

The confusing findings in Group C lambs can probably be ascribed to the limitations of the faecal examination (only 0,01 g of faeces was examined per count) but the possibility exists nevertheless that the maturation or fecundity of cryopreserved *N. spathiger* may be adversely affected by *per os* administration. In this respect it can be seen (Table 7) that significantly fewer ova ($P<0,01$) were present in worms from Group C (*per os* infestation) than in worms from Group B (intraduodenal administration). It is, however, unknown whether the uterine egg count correlates with egg production.

TABLE 8 Sheep: worm recovery data

Worm species \ Sheep	Group A—infested by injection						Group B—infested by injection*						Group C—infested *per os*					
	1	2	3	4	5	**Mean for the group**	6	7	8	9	10	**Mean for the group**	11	12	13	14	15	**Mean for the group**
H. contortus																		
L4	0	0	0	0	0	**0**	0	0	0	0	0	**0**	0	1	0	0	0	**0**
Adult	4 113	4 072	1 885	3 584	3 405	**3 412**	149	41	1	1	176	**74**	1 434	984	1 619	1 807	2 081	**1 585**
Total	4 113	4 072	1 885	3 584	3 405	**3 412**	149	41	1	1	176	**74**	1 434	985	1 619	1 807	2 081	**1 585**
Development (%)	43,0	42,6	19,7	37,5	35,6	**35,7%**	n**	n	n	n	n	**n**	15,0	10,3	16,9	18,9	21,8	**16,6%**
M. marshalli																		
L4	0	171	0	0	0	**34**	†	—	—	—	—	‡	—	—	—	—	—	‡
Adult	638	2 067	55	186	624	**714**	—	—	—	—	—	‡	—	—	—	—	—	‡
Total	638	2 238	55	186	624	**748**	—	—	—	—	—	‡	—	—	—	—	—	‡
Development (%)	5,3	18,7	0,5	1,6	5,2	**6,2%**	—	—	—	—	—	‡	—	—	—	—	—	‡
T. axei																		
L4	0	119	37	44	350	**110**	186	173	7	24	6	**79**	—	—	—	—	—	‡
Adult	1 778	2 743	6 667	1 807	1 717	**2 942**	1 057	1 559	1 808	239	651	**1 063**	—	—	—	—	—	‡
Total	1 778	2 862	6 704	1 851	2 067	**3 052**	1 243	1 732	1 815	263	657	**1 142**	—	—	—	—	—	‡
Development (%)	13,3	21,5	50,3	13,9	15,5	**22,9%**	9,3*	13,0*	13,6*	2,0*	4,9*	**8,6%***	—	—	—	—	—	‡
T. colubriformis																		
L4	0	0	0	35	1	**7**	0	1	2	27	0	**6**	0	0	0	0	0	**0**
Adult	374	378	375	394	268	**358**	526	532	277	788	652	**554**	227	388	346	282	82	**265**
Total	374	378	375	429	269	**365**	526	533	279	815	652	**560**	227	388	346	282	82	**265**
Development (%)	38,7	39,1	38,8	44,4	27,8	**37,7%**	n	n	n	n	n	**n**	n	n	n	n	n	**n**
O. columbianum																		
L4	137	99	94	312	453	**219**	—	—	—	—	—	‡	104	58	109	39	325	**127**
5 ths	130	4	146	14	39	**67**	—	—	—	—	—	‡	257	164	1 180	826	231	**532**
Total	267	103	240	326	492	**286**	—	—	—	—	—	‡	361	222	1 289	865	556	**659**
Development (%)	3,6	1,4	3,2	4,4	6,6	**3,8%**	—	—	—	—	—	‡	1,4	0,8	4,9	3,3	2,1	**2,5%**
O. circumcincta																		
L4	—	—	—	—	—	‡	30	0	0	1	0	**6**	9	5	0	0	0	**3**
Adult	—	—	—	—	—	‡	823	835	1 093	953	729	**887**	119	291	566	169	423	**314**
Total	—	—	—	—	—	‡	853	835	1 093	954	729	**893**	128	296	566	169	423	**317**
Development (%)	—	—	—	—	—	‡	38,4	37,6	49,2	42,9	32,8	**40,2%**	5,8	13,3	25,5	7,6	19,0	**14,3%**
T. falculatus																		
L4	—	—	—	—	—	‡	16	9	1	1	0	**5**	0	0	0	0	0	**0**
Adult	—	—	—	—	—	‡	1 216	553	180	269	765	**597**	37	63	149	274	361	**177**
Total	—	—	—	—	—	‡	1 232	562	181	270	765	**602**	37	63	149	274	361	**177**
Development (%)	—	—	—	—	—	‡	5,6	2,5	0,8	1,2	3,5	**2,7%**	0,0	0,1	0,2	0,3	0,4	**0,2%**
N. spathiger																		
L4	0	0	0	0	0	**0**	71	1	10	90	0	**34**	15	52	65	8	1	**28**
Adult	14	9	1	2	0	**5**	1 376	1 169	1 252	1 266	1 488	**1 310**	283	277	369	408	424	**352**
Total	14	9	1	2	0	**5**	1 447	1 170	1 262	1 356	1 488	**1 344**	298	329	434	416	425	**380**
Development (%)	n	n	n	n	n	**n**	27,1	21,9	23,7	25,4	27,9	**25,2%**	5,6	6,2	8,1	7,8	8,0	**7,1%**
C. ovina																		
L4	—	—	—	—	—	‡	0	10	413	59	0	**96**	—	—	—	—	—	—
5 ths	—	—	—	—	—	‡	420	485	380	613	747	**529**	—	—	—	—	—	—
Total	—	—	—	—	—	‡	420	495	793	672	747	**625**	—	—	—	—	—	—
Development (%)	—	—	—	—	—	‡	16,2	19,0	30,5	25,8	28,7	**24,0%**	—	—	—	—	—	—
D. filaria																		
Total (5ths)	—	—	—	—	—	‡	18	29	31	0	26	**21**	—	—	—	—	—	—
Development (%)	—	—	—	—	—	‡	0,5	0,8	0,9	0	0,7	**0,6%**	—	—	—	—	—	—

* Excepting for *T. axei* (infested *per os*)
** No pure cultures of these species dosed intentionally—these worms originated from mixed cultures (See Comment, Experiment 1)
† No larvae dosed, nor worms recovered
‡ Not applicable

TABLE 9 Calves: worm recovery data

Worm species \ Calf	Infestation by injection			Infestation *per os*		
	1	2	**Mean for group**	3	4	**Mean for group**
H. placei						
Total (adults)	1 260	2 013	**1 637**	261	185	**223**
Development (%)	11,8	18,9	**15,4**	2,4	1,7	**2,1**
O. ostertagi						
Total (adults)	860	2 499	**1 680**	318	56	**187**
Development (%)	19,8	57,5	**38,6**	7,3	1,3	**4,3**
Cooperia spp.						
Total (adults)	1 106	0	**553**	982	4	**493**
Development (%)	8,9	0,0	**4,0**	7,9	0,03	**3,5**
N. helvetianus						
Total (adults)	493	9 554	**5 024**	290	34	**162**
Development (%)	12,6	50,7	**22,1**	1,5	0,3	**1,0**
O. radiatum						
L4	75	2 216	**1 146**	2	0	**1**
5 ths*	1 487	37	**762**	0	1	**0,5**
Total	1 562	2 253	**1 908**	2	1	**1,5**
Development (%)	18,5	26,6	**22,5**	0,02	0,01	**0,02**

* Immature 5th stage worms

TABLE 10 Comparison of the survival of L3 of ovine nematodes frozen for various periods

Worm species	Survival (%)		
	After ±7,5* months	After ±23* months	After ±59** months
H. contortus	85,9	96,6	97,2
O. circumcincta	96,6	95,6	77,2
T. axei	98,0	96,7	97,6
T. colubriformis	100,0	93,5	95,9
N. spathiger	97,0	91,0	95,2
O. columbianum	83,3	87,3	88,9
C. ovina†	87,9	86,8	100,0

* See Van Wyk *et al.* (1977), Table 12 for the exact periods of storage
** See Table 1 (above) for times frozen
† L3 frozen for only 1, 16 and 52 months

TABLE 11 The survival of L3 of bovine nematodes after various periods of storage in liquid nitrogen*

Worm species	Survival (%)		
	1–5** months	23,5–27,6** months	55–59** months
H. placei	43,8	86,2	86,7
O. ostertagi	90,0	96,3	96,6
Cooperia spp.	89,2	97,2	97,1
N. helvetianus	99,2	98,6	97,2
O. radiatum	90,6	95,1	95,1

* The data refer to those L3 of which sufficient numbers were frozen in single batches so that the survival of each batch could be determined repeatedly
** See Van Wyk *et al.* (1977), Table 26, for the exact periods of storage

TABLE 12 Comparison of the infectivity of sheep nematode L3 frozen for various periods of time

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

* Excluding infestation *per os* and by intravenous injection
** See Van Wyk *et al.* (1977), Table 12, for the exact periods of storage
† L3 frozen for only 16 and 52 months

TABLE 13 Comparative development of bovine nematode L3 frozen for various periods of time*

Worm species	Development (%)†		
	After 1–5** months	After 23,5–27,6** months	After 55–59** months
H. placei	14,5	10,6	15,4
O. ostertagi	19,8	36,1	38,6
Cooperia spp.	23,1	0,4	8,9
N. helvetianus	3,5	12,0	12,6
O. radiatum	0,2	5,0	22,5
Mean development	**12,2**	**12,8**	**19,6**

* The data refer to those L3 of which sufficient numbers were frozen in single batches so that the survival of each batch could be determined repeatedly
** See Van Wyk *et al.* (1977), Table 26, for the exact periods of storage
† Excluding infestations *per os*

Worm development

Worm development in sheep infested by intravenous or gastro-intestinal inoculation was disappointingly low when compared to their development after 2 years of storage. *M. marshalli, T. falculatus, O. columbianum* and *D. filaria* developed particularly poorly. The percentage development of most species after 59 months of cryopreservation was similar to development after 7,5 months rather than to that after 23 months of storage (Table 12).

Nevertheless, sufficient numbers of each species developed after 5 years of storage to establish donor sheep.

Why *D. filaria* gave the poorest results warrants special consideration. Van Wyk *et al.* (1977), using a different batch of L3 from that used in the present trials, suggested that, even though ensheathed *D. filaria* L3 survived freezing very well, exsheathed L3 developed more effectively when thawed. Those used in the present trial were exsheathed, but development was very poor. Intravenous infestation was used because in an earlier trial this route was more effective than inocu ation into the duodenum (Van Wyk, unpublished data, 1976). Recently, however, *D. filaria* was established in sheep by intravenous injection of the same batch of L3 used in the present trial. After 2 sheep had each been injected intravenously with 11 000 live L3, 400 000 infective larvae were recovered from their faeces for replenishing supplies of cryopreserved L3 (Van Wyk, unpublished data, 1979).

Development of all the worm species in calves (Table 13) was better than before, but was still lower than in sheep.

Calves 2 and 4 were given L3 *Cooperia* spp. which had been stored for only 40 months because too few of the original batch (stored for 59 months) were left over for infesting all 4 calves. The second batch developed extremely poorly (Table 9), despite the fact that survival and activity after thawing were similar in the 2 batches. This is ascribed to the unexplained variability in survival and infectivity of different batches of larvae of a given species (Kelly & Campbell, 1974; Van Wyk *et al.*, 1977) rather than to the possibility of variations between the calves in their resistance to *Cooperia*.

A surprising result of this trial was the development of all worm species dosed *per os* to the sheep and calves. *O. radiatum* was an exception, however, whose results give cause for inquiry. This species was recovered in such small numbers from the 2 calves infested *per os* that cross-transfer from other calves at necropsy should be considered as a possibility. Development of all species infested *per os* was much lower than with infestation by injection into the abomasum or duodenum, but even *T. colubriformis* developed in this group of sheep, although Campbell & Thomson (1973) were unable to infest sheep *per os* with either frozen or unfrozen exsheathed *T. colubriformis*. Van Wyk *et al.* (1977) did not kill the single sheep in which *T. falculatus* was administered *per os* but relied on differential larval counts. In the present studies, however, its development was confirmed post-mortem.

Some of the stored larvae used in the previous (Van Wyk *et al.*, 1977) and the present trials were contaminated with other species. In Group A, for example, a few *N. spathiger*, which originated from the *M. marshalli* cultures known to contain a low percentage of *Nematodirus* L3, developed; and in Group B *H. contortus* and *T. colubriformis* developed from larvae from an *H. contortus* donor. Van Wyk *et al.* (1977) showed that the standard cultures of *T. falculatus* at this laboratory were probably contaminated with *H. contortus*, while those of *T. axei* were probably contaminated with *T. colubriformis*. In the present trial microscopic examination of the L3 after thawing revealed that the *O. circumcincta* cultures, too, were contaminated with *T. colubriformis*. Thus the *T. colubriformis* infestations in Group C (*per os* infestation) were unintentional.

Conclusions

This appears to be the first time that nematode larvae have been tested for infectivity after storage in the gas phase of liquid nitrogen for up to 59 months and that *O. circumcincta, T. colubriformis, N. spathiger, O. columbianum, H. placei, O. ostertagi, N. helvetianus* and possibly *O. radiatum* have been shown to be infective by *per os* infestation and *D. filaria* by intravenous injection after cryopreservation in the gas phase of liquid nitrogen.

The prolonged survival of L3 *H. contortus, O. circumcincta, T. axei, T. colubriformis, N. spathiger, O. columbianum, C. ovina, H. placei, O. ostertagi, N. helvetianus, Cooperia* spp. and *O. radiatum* in the gas phase of liquid nitrogen, coupled with adequate development in the definitive hosts after thawing, should dispel any doubts concerning the practicability of this method for routine use in the laboratory.

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