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BENZIMIDAZOLE-RESISTANT *HAEMONCHUS CONTORTUS*—THE EFFECT OF CRYOPRESERVATION ON THE RESISTANCE OF SUCCESSIVE GENERATIONS

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ABSTRACT

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Infective larvae (L3) of a strain of benzimidazole-resistant *Haemonchus contortus* were stored in the gas phase of liquid nitrogen, thawed and used to infest worm-free sheep from which, in turn, larvae were cultured, frozen and thawed. This cycle was repeated 5 times. Thereafter, the progeny of the cryopreserved larvae were compared with the progeny of the original untreated larvae for susceptibility to benzimidazole anthelmintics.

Repeated freezing of consecutive generations of L3 in liquid nitrogen did not appear to affect their relative resistance to benzimidazoles and, although other strains must also be tested, it would seem that cryopreservation may be used for storing resistant strains.

Résumé

HAEMONCHUS CONTORTUS *RÉSISTANT AU BENZIMIDAZOLE—L'EFFET DE LA CRYOCONSERVATION SUR LA RÉSISTANCE DES GÉNÉRATIONS SUCCESSIVES*

*Des larves infectieuses (L3) d'une souche d'*Haemonchus contortus *résistante au benzimidazole ont été placées dans la phase gazeuse d'azote liquide, puis dégelées et utilisées pour infester des moutons indemnes d'infestation parasitaire et, de là, des larves furent ensuite cultivées, gelées et dégelées. Ce cycle fut répété 5 fois. Ensuite, les descendants des larves cryoconservées furent comparées avec la descendance des larves originales non traitées en ce qui concerne leur susceptibilité aux anthelmintiques de benzimidazole.*

La congélation répétée des générations consécutives de L3 dans l'azote liquide ne sembla pas affectér leur résistance relative au benzimidazole et, bien que d'autres souches doivent également être éprouvées, il semblerait que la cryoconservation puisse être utilisée pour la conservation des souches résistantes.

INTRODUCTION

Campbell, Blair & Egerton (1972; 1973), Campbell & Thomson (1973), Rose (1973), Van Wyk, Gerber & Van Aardt (1977) and Van Wyk & Gerber (1980a) successfully preserved exsheathed infective larvae (L3) of most of the common gastro-intestinal nematodes of ruminants in liquid nitrogen, with little loss in viability. When larvae were thawed after as long as 56 months of cryopreservation, more than 90% of the larvae were usually alive and some were infective (Van Wyk *et al.*, 1977; Van Wyk & Gerber, 1980a). The technique could therefore be used for storing different strains of nematodes (Van Wyk *et al.*, 1977).

Strains of *Haemonchus contortus* in South Africa, as elsewhere in the world, have become resistant to the benzimidazole anthelmintics (Berger, 1975; Le Jambre, 1978). There has also been an instance recently of resistance to rafoxanide (Van Wyk & Gerber, 1980b). It was decided to store reference strains of resistant *H. contortus* for use in testing the efficacy of new benzimidazole remedies coming on the market and for basic research.

Van Wyk *et al.* (1977), discussing the problems encountered in maintaining strains of nematode parasites in sheep, included among them the possibility of selection within the strains due to serial passage under artificial conditions, the costs of maintaining donor animals and the danger of cross-contamination with the consequent loss of a strain.

Cryopreservation seemed to be a possible means of solving these problems, provided that the characteristics of resistance of the nematode strains were unaffected. The following trials were carried out to compare the susceptibility of the progeny of cryopreserved and unfrozen benzimidazole-resistant *H. contortus*.

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MATERIALS AND METHODS

Strain of H. contortus

The strain of *H. contortus* used in the investigations originated from Boshof in the Orange Free State (Berger, 1975) and was passaged once in the laboratory by Berger (Coopers, Kwanyanga, East London) before being used in this investigation.

Comparison of the progeny of unfrozen and frozen larvae

The Boshof strain of *H. contortus* was maintained in donor sheep, using larvae never exposed to cryopreservation in liquid nitrogen ("unfrozen substrain"). In addition, some larvae of the strain were frozen for later comparison of their progeny ("frozen substrain") with the progeny of the unfrozen substrain.

(a) *Frozen substrain* (Table 1)

Larvae of the Boshof strain of *H. contortus* were frozen in the gas phase of liquid nitrogen (Van Wyk *et al.*, 1977) and were subsequently thawed and used to

TABLE 1 Cryopreservation of consecutive generations of the Boshof (Berger, 1975) strain of *H. contortus*

Worm generation	Larvae (L3)			
	Date frozen	Days frozen	Alive (%)	Infective dose (live larvae)
I.....	1976.10.13	8	88,1	3 568
II.....	1976.12.22	28	95,9	7 253
III.....	1977.02.18	13	89,0	6 870
IV.....	1977.04.07	57	64,0	6 400
V.....	1977.07.13	55	49,0	45 000*

* The sheep infested with these L3, in turn yielded L3, which were neither exsheathed nor cryopreserved before being used in the experiment (Table 3 and p. 143, column 2, paragraph 2 of the text)

infest a sheep. Larvae from this sheep were frozen, thawed and used for infesting a further sheep, until the cycle had been repeated 5 times.

Larvae obtained from the last passage were not frozen before being used in the trial.

(b) *Unfrozen substrain*

The substrain not exposed to cryopreservation was passaged in donor sheep only when a low faecal egg count made it necessary. The last passage was made shortly before the commencement of the trial to ensure that faeces with a high egg count were available.

Infective larvae and infestation

The L3 of each substrain which were younger than 3 weeks at infestation, were cultured in larval hatching chambers (Van Wyk *et al.*, 1977) and stored at 4 °C, using the technique of Reinecke (1973).

While the predecessors of some of the larvae had been exposed to cryopreservation, none of the larvae used in the trial were frozen immediately before infestation.

Experimental animals

The Dorper sheep (donor sheep) used for the passage of the frozen and unfrozen substrains were raised worm-free and were treated with levamisole* at 15–20 mg/kg as a further precaution at least 3 days before being infested.

The sheep used in the trial for comparing the 2 substrains were of mixed breeds and sexes (Table 2). Apart from the Merino rams, which were raised on pasture, all the others were born and raised on concrete under conditions of minimal exposure to worms. Before the commencement of the trial, all the sheep were drenched twice with levamisole at 15 mg/kg live mass, whereafter faeces collected at random from 20 of the 62 sheep were free from nematode ova.

The experimental animals were allocated to the different treatments (see Experimental Design below) as follows: Prior to the commencement of the trial, the mass of the sheep were determined, ranked

TABLE 2 Experimental animals

Sheep		Substrain of *H. contortus*	
Breed	Sex	Frozen	Unfrozen
Merino.....	♂	14	15
Dorper.....	♂	4	4
Dorper.....	♀	12	11
Merino.....	♀	1	1
Total.....	—	31	31

(separately for each breed and sex) according to live mass and divided into 2 groups of 31 sheep, using tables of random numbers. On the day of treatment (Day 0), one sheep from each group was selected as a Day 0 worm control, and the mass of the others was again determined, they were ranked separately as before and, using tables of random numbers, each group was sub-divided into 3 groups of 10 animals and the 6 subgroups thus formed were allocated to the various treatments (Table 3).

Experimental design

Details of infestation, treatment and slaughter of the animals are listed in Table 3. The 6 groups, each of 10 sheep, were infested from Day −24 to Day −22 with a total of 3 384 larvae, 3 groups receiving larvae of the frozen substrain and 3 groups larvae of the unfrozen substrain. On Day 0 4 groups (2 of each substrain) were treated with either thiabendazole or mebendazole, while the other 2 groups remained as untreated control groups. Two sheep killed on Day 0 acted as controls for the development of the worms.

On Day +21 and +23 all the sheep were killed for worm recovery.

Thiabendazole (Thibenzole, MSD, 14,67% m/v batch No. May 75 S. 1 582) and mebendazole (Multispec, Ethnor, 5% m/v batch No. 21 S 406) were used to treat the animals, as Berger (1975) had reported a high degree of resistance to thiabendazole, while resistance to mebendazole was not as marked.

TABLE 3 Experimental design

Date	Day	Number of L3 dosed	
		Frozen substrain*	Unfrozen substrain*
1977.10.20	−24	1 128	956
1977.10.21	−23	1 128	956
1977.10.22	−22	1 128	1 472
	Total L3	3 384	3 384
1977.11.14	0	Sixty sheep weighed, Groups A & B being treated with thiabendazole (44 mg/kg) and Groups C & D with mebendazole (20 mg/kg); Groups E & F remained as untreated controls. Killed 2 sheep (1 for each sub-group of larvae) as Day 0 worm controls	
1977.12.05	+21	Killed Group B (frozen, treated with thiabendazole) + Group A (unfrozen, treated with thiabendazole) + Group C (unfrozen, treated with mebendazole)	
1977.12.07	+23	Killed Group D (frozen, treated with mebendazole) + Group F (frozen, untreated controls) + Group E (unfrozen, untreated controls)	

* None of the L3 used for the final infestation in this experiment was cryopreserved; the group designated cryopreserved originated from consecutive generations of L3, each generation of which was frozen for a short period before being thawed for infestation (Table 1)

* Ripercol (Ethnor)

Worm recovery and estimation of worm burdens

The abomasa were collected at necropsy, as described by Reinecke (1973). While the worms were recovered from the abomasal ingesta by migration, using a modification of the gelled-agar technique of Van Wyk & Gerber (1978), Van Wyk (1978), and Van Wyk, Gerber & Groeneveld (1980), the abomasal mucosae were digested for worm recovery, as described by Reinecke (1973).

The supernatant was poured off before the ingesta were gelled in agar and the samples containing the worms which had migrated from the agar slabs and 8/10 of the abomasal ingesta recovered from the agar slabs were examined macroscopically for worms. Total worm counts of the mucosal digests and of the remaining $2\times1/10$ aliquots of the ingesta were done microscopically.

In each sample only the first 25 worms recovered (or 50 for those samples in which nematode larvae occurred) were identified, but if fewer than 25 (or 50 in samples containing larvae) were recovered, they were all identified (Reinecke, 1973).

Statistical evaluation

The worm burdens of the 2 untreated control groups of sheep were compared by means of the Mann-Whitney U-test (Siegel, 1956).

Because a highly significant difference in worm development was found in the groups of untreated controls, the method of Dunn (Miller, 1966) was used to compare the worm burdens of the sheep treated with anthelmintics (Group A *v.* Group B and Group C *v.* Group D), since this method takes into consideration the difference between the control groups when the treated ones are compared statistically.

RESULTS

Resistance to anthelmintic treatment (Tables 4, 5 & 6)

Significantly more worms ($P=0,01$) were recovered from the untreated control group infested with the frozen substrain than from the controls of the unfrozen substrain (a mean of respectively 55,4% and 38,3% development of infective larvae per sheep).

Thiabendazole removed a mean of 10,7% of the frozen and 9,5% of the unfrozen substrain, while mebendazole removed 48,0% and 59,0% respectively. The differences between the frozen and unfrozen groups were not significant for either anthelmintic when they were compared by the method of Dunn (Miller, 1966).

The mean percentages of the 4th stage worms (L4) recovered are listed in Table 6. Highly significantly more L4 of the unfrozen substrain were recovered than of the frozen substrain ($P<0,001$).

TABLE 4 The numbers of worms recovered and the percentages which consisted of L4

Sheep No.	Groups (substrains)											
	Thiabendazole				Mebendazole				Untreated controls			
	A. Unfrozen		B. Frozen		C. Unfrozen		D. Frozen		E. Unfrozen		F. Frozen	
	Total worms	L4 (%)	Total worms	L4 (%)	Total worms	L4 (%)	Total worms	L4 (%)	Total worms	L4 (%)	Total worms	L4 (%)
1.....	1 402	5,7	2 134	0	522	8,4	976	0	1 058	2,7	1 962	0,1
2.....	1 329	2,8	2 284	0	217	17,1	739	17,6	1 423	2,0	2 161	0
3.....	559	2,3	501	0	702	27,6	999	0	2 315	0	2 399	0
4.....	1 216	0,9	2 661	0,4	133	39,9	1 812	0	979	1,3	2 411	0,1
5.....	1 654	0,4	1 157	0	336	18,5	773	0	2 001	1,0	2 075	0
6.....	1 210	10,5	706	0	691	10,6	429	0	1 236	1,1	37	0
7.....	1 003	3,5	1 942	0,3	511	51,3	1 817	0	1 187	2,8	2 353	0
8.....	1 535	2,6	1 969	0,1	875	17,0	643	4,7	773	0,8	1 503	0
9.....	1 219	3,5	2 073	0,7	940	9,5	1 079	0	1 283	2,4	1 789	1,1
10.....	592	12,5	1 308	0	388	40,5	473	0,4	695	4,5	2 057	0
Mean.....	1 171,9	4,5	1 673,5	0,2	531,5	24,0	974,0	2,3	1 295,0	1,9	1 874,7	0,1
±SD*.....	363,3	4,0	714,2	0,3	270,1	15,1	491,9	5,6	512,0	1,3	705,3	0,4

* Standard deviation

TABLE 5 The mean number of worms that developed in each group (expressed as a percentage of the number of larvae dosed)

Treatment	Substrain	
	Frozen (%)	Unfrozen (%)
Untreated controls.....	55,4 (±21)	38,3 (±15)
Thiabendazole.....	49,5 (±21)	34,6 (±11)
Mebendazole.....	28,8 (±15)	15,7 (± 8)

TABLE 6 Numbers of worms in the 4th stage expressed as a percentage of the total numbers recovered

Treatment	Substrain	
	Frozen	Unfrozen
Untreated controls.....	0,1 (±0,4)	1,9 (±1,3)
Thiabendazole.....	0,2 (±0,3)	4,5 (±4,0)
Mebendazole.....	2,3 (±5,6)	24,0 (±15,1)

Discussion

The experimental design for these trials provided for the simultaneous investigation of the effect of freezing on the progeny of the larvae of 2 strains of *H. contortus*. Unfortunately, in the case of the second strain (designated the OP-M strain, isolated at Onderstepoort and more resistant to benzimidazoles than the Boshof strain), a susceptible strain of *H. contortus* was used by mistake in the controls instead of the resistant unfrozen substrain. Although the frozen substrain of this Onderstepoort strain apparently reacted like the corresponding unfrozen substrain (tested on a small scale prior to this experiment—Van Wyk, unpublished data, 1976), the detailed results, though not reported in this paper, were used for detailed evaluation of the gelled-agar method of worm recovery (Van Wyk *et al.*, 1980).

Because significantly more larvae of the frozen substrain developed in the untreated sheep than larvae of the unfrozen substrain (P=0,01), comparison of the pairs of groups treated with either thiabendazole, or mebendazole was complicated. By using the method of Dunn (Miller, 1966), however, it could be shown that the differences in reaction of the frozen and unfrozen substrains to the 2 anthelmintics were not significant.

Because Berger (1975) reported a 57,9% development of the unfrozen Boshof strain in untreated control sheep, it is unlikely that the differences in development in the present experiment between the frozen Boshof substrain (55,4%) and the unfrozen substrain (38,3%) are due to an innate difference caused by exposure of a series of generations of L3 to cryopreservation. It seems more likely that the difference was due to the usual variations in the infectivity of different batches of L3, but this cannot be confirmed from the present study.

From the percentages of live larvae recovered after thawing of the IVth and Vth generations of the frozen substrain (Table 1), it would appear that repeated exposure of successive generations of L3 reduced the capacity of larvae to survive in liquid nitrogen. Although this possibility cannot be excluded, it must be pointed out that previous workers have found considerable variability in different batches of cryopreserved L3 (Kelly & Campbell, 1974; Van Wyk *et al.*, 1977). Amongst others, Van Wyk *et al.*, (1977) obtained unsatisfactory cryopreservation with an earlier isolate of the OP-M strain not previously exposed to freezing in liquid nitrogen. On another occasion these workers obtained only 52,9% survival after short-term storage of larvae of an unfrozen benzimidazole-susceptible strain of *H. contortus*.

One realises that insufficient repeat experiments have been made for the results to be absolutely conclusive, but the present results seem to justify the conclusion that the use of liquid nitrogen for cryopreservation after exsheathment of the L3 is suitable for storing strains of resistant worms. This should prove to be a better safeguard against loss of the strain than maintaining it in donor animals alone. Furthermore, there seems no reason to doubt that storage of worm strains with other specifically-required characteristics may be equally suitable. This should, however, be confirmed experimentally.

No explanation can be given for the fact that highly significantly more of the unfrozen substrain larvae failed to develop further than the L4 stage than with the frozen substrain.

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