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# METHODS OF INFESTING SHEEP WITH GASTRO-INTESTINAL NEMATODES AFTER CRYOPRESERVATION: DOSING OF LARVAE IN GELATIN CAPSULES COMPARED TO DOSING OF LARVAE IN WATER SUSPENSION

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## ABSTRACT

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Cryopreservation of the infective larvae ($L_3$) of nematodes is being used increasingly for the routine maintenance of pure strains of nematodes in the laboratory. Gelatin capsules are frequently used to administer the $L_3$ of nematodes to sheep, but with some nematode species this method usually does not give good results with cryopreserved larvae.

The development in sheep of cryopreserved $L_3$ of *Trichostrongylus* spp. and other ovine nematodes was compared when the larvae were administered either in a suspension or in gelatin capsules with or without the use of $CuSO_4$ to stimulate the oesophageal groove reflex.

Significantly larger numbers of cryopreserved $L_3$ developed when dosed *per os* in suspension than when the $L_3$ were dosed in gelatin capsules. Stimulation of the oesophageal groove did not appear to affect the numbers of worms that developed from $L_3$ dosed in suspension.

It is speculated that $L_3$ in suspension bypass the rumen to go directly into the abomasum, while those in gelatin capsules enter the rumen, thus closely approximating the natural infestation of grazing ruminants. In these trials, however, only cryopreserved $L_3$ were used.

Sufficient numbers of cryopreserved $L_3$ of *Trichostrongylus falculatus* and *T. colubriformis* in suspension developed, so that it seems unlikely that laparotomy will be required for routine infestations in the laboratory.

## INTRODUCTION

In some of the modern anthelmintic tests, such as the larval test using the NPM statistical analysis of Groeneveld & Reinecke (1969), numerous doses of nematode infective larvae ($L_3$) are administered to experimental animals.

To facilitate the handling of multiple doses, the $L_3$ are often concentrated on filter paper and placed in gelatin capsules before being dosed to the animals (Reinecke, 1973). The alternative, i.e. dosing the $L_3$ in water suspension, is clumsier and more time-consuming and involves numerous test tubes and syringes, etc.

During the past decade cryopreservation of $L_3$ in liquid nitrogen has come into use to obviate dependence on donor animals alone for the various species and strains of nematodes maintained in the laboratory. Unfortunately, when some nematodes, such as *Trichostrongylus* spp. which inhabit the small intestine, are cryopreserved and then thawed, their infectivity is either nil (Campbell & Thomson, 1973) or poor (Van Wyk, Gerber & Van Aardt, 1977). The latter authors overcame this by depositing the $L_3$ directly in the abomasum or duodenum during laparotomy.

Laparotomy, being time-consuming, reduces the value of using cryopreserved larvae as a routine technique. In a search for alternative methods, various techniques of infestation were tested, including infestation with the $L_3$ in suspension, or in gelatin capsules with and without pre-stimulation of the oesophageal groove with $CuSO_4$.

## MATERIALS AND METHODS

### *Infective material*

The isolation and maintenance of the various pure strains of nematodes used in the investigations have been described (Van Wyk *et al.*, 1977). Cryopreservation and thawing of the $L_3$ occurred as described by Van Wyk & Gerber (1980a).

All the *Trichostrongylus falculatus* $L_3$ used in the investigations orginated from the same batch of $L_3$, frozen on 19 October 1979, by which date this strain had been passaged only 3 times in the laboratory, twice without, and once with cryopreservation.

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### *Experimental animals*

Dorper sheep, born and housed on concrete under conditions of minimal exposure to worms, were used in the investigations. As an added precaution, the sheep were drenched 4–10 days before the commencement of the trial with either levamisole or fenbendazole* at dosages varying from 2–3 times those recommended by the manufacturers.

The ages and sexes of the sheep are listed in each experiment.

The animals, apart from those used in the preliminary trial and 4 sheep from each of Experiments III and IV, were allocated to the experimental groups, using tables of random numbers.

### *Infestation of the sheep*

The sheep were infested using either of the following methods:

(a) *Gelatin capsules*. Aliquots of known concentrations of $L_3$ were deposited on filter-paper discs, which were then rolled up, placed in gelatin capsules and dosed to the sheep with a balling gun (Reinecke, 1973).

(b) *$L_3$ in suspension*. Similar aliquots of $L_3$ in test-tubes were mixed and drawn up in plastic syringes and then administered either *per os* or by injection into the rumen, as described in Experiments 1–4.

### *Worm recovery, counts and identification*

Intestinal ingesta were gelled in agar for worm recovery (Van Wyk, Gerber & Groeneveld, 1980). In addition, the mucosae of the organs concerned were digested as described by Reinecke (1973).

Apart from the residual ingesta after migration of the worms from the agar gels (in which a minimum of 10 % of each sample was examined for worms), total counts were done of the worms in all the experimental samples. Of the 80 266 worms gelled in the agar, 97,9 % migrated successfully from the slabs.

In each sample the first 50 worms recovered were identified, but if fewer than 50 worms were recovered, all were identified (Reinecke, 1973).

* Ripercol (Janssen) and Panacur (Hoechst)

*Statistical analysis*

In each trial worm burdens of the different groups were compared using the Mann-Whitney U Test (Siegel, 1956).

*Definitions*

(a) Infestation of sheep with *frozen* or *cryopreserved* $L_3$ means that $L_3$ were exsheathed, frozen in liquid nitrogen and thawed (Van Wyk *et al.*, 1977) before being dosed to the sheep.

(b) When $L_3$ were administered in suspension, aliquots of known concentrations of $L_3$ in 0,09 % NaCl in test-tubes were mixed thoroughly and drawn into hypodermic syringes immediately before being administered either *per os* or by injection into the rumen, as described in each trial.

EXPERIMENTAL PROCEDURES

***Experiment I.* (Preliminary trial). $L_3$ in suspension administered *per os* after pre-stimulation with $CuSO_4$ of the oesophageal groove reflex**

In previous experiments, when cryopreserved $L_3$ of *T. colubriformis* and *T. falculatus* were administered to sheep either *per os* or by injection into the rumen (Table 1) (Campbell & Thomson, 1973; Van Wyk *et al.*, 1977; Van Wyk & Gerber, 1980a), there was either no development, or else very poor development.

The poor results after infestation *per os* could theoretically have resulted from the entrance of the $L_3$ into the rumen, implying that exsheathed cryopreserved $L_3$ of these species need to bypass the rumen if they are to have a reasonable chance of survival. This supposition is supported by the fact that more worms develop when the $L_3$ are placed directly in the duodenum. Consequently, it was decided to attempt to bypass the rumen by pre-stimulation with $CuSO_4$ immediately before infestation.

*Method*

Two 10-month old Dorper ewe lambs were each infested daily for 3 days with 20 300 $L_3$ of *T. falculatus* administered *per os*, and were slaughtered 77 days later for worm recovery.

The $L_3$ were dosed in suspension 2 min after 5 mℓ of a 10 % solution of $CuSO_4$ had been dosed *per os* to each sheep.

*Results*

The maximum *T. falculatus* faecal egg counts (epg) of the 2 sheep were 5 700 and 7 200 respectively, and the percentages of development of *T. falculatus* were 29,4 % and 30,5 %.

*Comment*

The percentage development of the cryopreserved *T. falculatus* $L_3$ in these 2 lambs was higher even than when they had previously been placed directly into the duodenum (Table 1). In previous experiments with frozen $L_3$ administered by similar routes, fewer *T. falculatus* than *T. colubriformis* developed. In this trial however, the development was much better than that previously obtained with *T. colubriformis* when dosed *per os*.

There were no controls in this trial, but it is possible that either the pre-stimulation with $CuSO_4$ or the very young age of these lambs could have been responsible for the exceptionally good results.

TABLE 1 Previous publications: Percentage development of cryopreserved *Trichostrongylus* spp. $L_3$ administered by different routes to sheep

| Species of *Trichostrongylus* | Route of administration | | |
|---|---|---|---|
| | Abomasum or duodenum | *Per os* | Rumen |
| *T. colubriformis* | 8,8[1]* <br> 10,6[1] <br> 45,5[1] <br> 62,7[1] <br> 37,7[2] | 8,3[1] | 0[3] |
| *T. falculatus* | 4,3[1] <br> 2,7[2] | 0,2[2] | — |

* The figures in brackets represent the following:

[1] Van Wyk *et al.* (1977)

[2] Van Wyk & Gerber (1980)

[3] Campbell & Thomson (1973)

***Experiment II.* Comparison of the development of various nematodes in lambs and adult sheep, with or without pre-stimulation with $CuSO_4$**

*Method*

Four lambs, 15–16 weeks old at the commencement of the trial, and 4 adult sheep, 57–61 weeks of age, were used. Two of the lambs and 2 of the ewes were pre-stimulated with $CuSO_4$, but not the other 4 sheep.

The pre-treatment of the $L_3$ and the numbers dosed per sheep are summarised in Table 2. The $L_3$ were in suspension and were administered daily over 3 days, the sheep being killed for worm recovery 31 days after the last infestation.

TABLE 2 Experiment II: Pretreatment of $L_3$ and the numbers dosed to sheep

| Worm species | Infective larvae ($L_3$) | | |
|---|---|---|---|
| | Months frozen | Alive (%) | No. (alive) dosed per sheep* |
| *T. falculatus* | 34 | 100 | 24 600 |
| *M. marshalli*** | 74 | 15 | 2 100 |
| *O. columbianum* | 42 | 95 | 4 125 |
| *N. spathiger* | 61 | 94 | 2 190 |

* Administered in 3 doses of similar size: one dose per day for 3 days

** Contaminated with a low percentage of *H. contortus*

*Results*

The numbers of nematodes recovered from these sheep are listed in Table 3.

Except for *H. contortus* ($P<0,02$), the differences in worm burdens between the groups of sheep pre-stimulated with $CuSO_4$ and those that were not, were not significant ($P>0,2$–$P>0,5$).

The *Marshallagia marshalli* burdens of the lambs were significantly larger than those of the adult sheep ($P<0,02$), while the burdens of *T. falculatus* and *Oesophagostomum columbianum* were nearly significantly different ($P=0,57$). For the other 2 worm species the differences were not significant.

*Comment*

Although it must be kept in mind that very few sheep were used in these trials, it seems unlikely that pre-stimulation with $CuSO_4$ had a favourable effect on the development of the cryopreserved $L_3$.

The question therefore arose as to why the frozen $L_3$ of *T. falculatus* developed so much better in these 2 trials than they did in previous trials. One possible explanation

TABLE 3 Experiment II: Numbers of nematodes recovered

| Sheep and treatment | *T. falculatus* | *M. marshalli* | *O. columbianum* | *N. spathiger* | *H. contortus* |
|---|---|---|---|---|---|
| *With $CuSO_4$* | | | | | |
| A* 1 | 3 615 | 1 | 270 | 830 | 18 |
| A 2 | 4 314 | 0 | 127 | 85 | 36 |
| L 3 | 7 198 | 185 | 319 | 16 | 12 |
| L 4 | 4 102 | 22 | 184 | 339 | 17 |
| Mean | 4 807 | 52 | 225 | 318 | 21 |
| % | 19,5 | 2,5 | 5,5 | 14,5 | — |
| *Without $CuSO_4$* | | | | | |
| A 5 | 1 908 | 0 | 20 | 44 | 57 |
| A 6 | 4 696 | 0 | 63 | 61 | 41 |
| L 7 | 6 096 | 54 | 214 | 214 | 64 |
| L 8 | 7 640 | 41 | 313 | 384 | 52 |
| Mean | 5 085 | 24 | 153 | 176 | 54 |
| % | 20,7 | 1,1 | 3,7 | 8,0 | — |
| | P>0,3 | P>0,5 | P>0,2 | P>0,5 | P<0,02 |
| *A vs L* | | | | | |
| Mean A | 3 633 | 0,3 | 120 | 255 | 38 |
| Mean L | 6 259 | 76 | 257 | 238 | 36 |
| | P<0,1 | P<0,02 | P<0,1 | P>0,4 | P>0,4 |

* A represents adult sheep: L represents lambs

TABLE 4 Experiment III: Numbers of nematodes recovered from sheep predosed with $CuSO_4$ compared to those from undosed sheep

| Groups | *T. falculatus* | | *T. colubriformis* | |
|---|---|---|---|---|
| | Sheep No. | No. of worms | Sheep No. | No. of worms |
| *With $CuSO_4$* | | | | |
| Suspension | 9 | 2 667 | 17 | 6 235 |
| Suspension | 10 | 3 230 | 18 | 2 634 |
| Capsules | 11 | 546 | | |
| Capsules | 12 | 1 989 | | |
| Mean | | 2 108 | | — |
| *Without $CuSO_4$* | | | | |
| Suspension | 13 | 2 047 | 19 | 6 740 |
| Suspension | 14 | 1 965 | 20 | 9 022 |
| Capsules | 15 | 736 | | |
| Capsules | 16 | 459 | | |
| Mean | | 1 302 | | — |
| | | P=0,17 | | — |

is that, while the $L_3$ were administered in suspension in these 2 trials, Van Wyk *et al.* (1977) and Van Wyk & Gerber (1980a) made use of gelatin capsules in all cases in which $L_3$ were dosed *per os* (Van Wyk, unpublished data, 1980). It was therefore decided to compare $L_3$ in suspension with $L_3$ in gelatin capsules and to confirm the above results with $CuSO_4$ in a further tiral.

***Experiment III.* Further comparison of gelatin capsules and suspension for administering *T. falculatus* and of the effect of pre-stimulation of the oesophageal groove reflex on the development of *T. falculatus* and *T. colubriformis***

*Method*

Eight ewes, varying in age from 4–5 months, were each infested over 3 days with a total of 7 100 $L_3$ of *T. falculatus* in 3 similar doses. Four 4 month-old wethers were likewise infested with a total of 15 666 $L_3$ of *T. colubriformis*. The form in which the $L_3$ were administered (suspension or capsules) is noted in Table 4.

One hundred per cent of the 400 $L_3$ of both the *T. falculatus* (frozen for 37 months) and *T. colubriformis* (frozen for 8 months) were alive when examined for viability after thawing.

The sheep were killed for worm recovery 26 days after the last infestation.

*Results*

The results are summarised in Tables 4 & 5.

As was the case in Experiment II, the *T. falculatus* burdens of the sheep pre-stimulated with $CuSO_4$ before infestation, did not differ significantly from those of the untreated group (P = 0,17; Table 4).

By contrast, the *T. falculatus* burdens of the sheep that were infested with $L_3$ in suspension did differ significantly from those that were infested with $L_3$ in capsules (P<0,03; Table 5).

In the case of *T. colubriformis*, too few sheep were used for meaningful statistical comparison of groups with or without pre-stimulation, but the worm burdens also did not appear to differ appreciably. Furthermore, the percentages of development of the *T. falculatus* and *T. colubriformis* did not differ significantly (P>0,3).

*Comment*

While $CuSO_4$ pre-stimulation once again did not influence worm development significantly, it is clear that the $L_3$ in suspension developed much better than did those in gelatin capsules.

The percentages of development of *T. falculatus* and *T. colubriformis* are, strictly speaking, not statistically comparable, because the sheep were not allocated at random to the 2 experimental groups. Nevertheless, the similarity in development (34,9 % and 39,3 %, respectively, when both were administered in suspension) was

TABLE 5 Experiment III: Numbers of worms recovered when $L_3$ were dosed either in suspension or in gelatin capsules, with or without pre-stimulation with $CuSO_4$

| Groups | *T. falculatus* | | | *T. colubriformis* | | |
|---|---|---|---|---|---|---|
| | Sheep No. | No. of worms | %* | Sheep No. | No. of worms | %* |
| *Suspension* | | | | | | |
| With† | 9 | 2 667 | 37,6 | 17 | 6 235 | 39,8 |
| With | 10 | 3 230 | 45,5 | 18 | 2 634 | 16,8 |
| Without | 13 | 2 047 | 28,8 | 19 | 6 740 | 43,0 |
| Without | 14 | 1 965 | 27,7 | 20 | 9 022 | 57,6 |
| Mean | • | 2 477 | 34,9 | • | 6 158 | 39,3 |
| | • | • | • | • | • | P>0,3 |
| *Capsules* | | | | | | |
| With | 11 | 546 | 7,7 | | | |
| With | 12 | 1 989 | 28,0 | | | |
| Without | 15 | 736 | 10,3 | | | |
| Without | 16 | 459 | 6,4 | | | |
| Mean | • | 933 | 13,1 | | | |
| | • | P<0,03 | • | | | |

* Percentage development

† With or without pre-stimulation with $CuSO_4$ (see Table 4)

unexpected in the light of previous reports, which listed 0,2 % development of *T. falculatus* and 8,3 % of *T. colubriformis*, when both were administered *per os* in gelatin capsules (Table 1). Even when $L_3$ were administered in gelatin capsules, the development of *T. falculatus* in the present trial (13,1 %) was better than the 8,3 % development of *T. colubriformis* recorded previously. This is considered more fully in the discussion below.

TABLE 6 Experiment IV: Numbers of worms recovered when $L_3$ were dosed either in suspension or in gelatin capsules

| Group and Sheep No. | *T. falculatus* (No.) |
|---|---|
| *Suspension* | |
| 21 | 1 581 |
| 22 | 976 |
| 23 | 466 |
| 24 | 1 007 |
| Mean | 1 008 |
| *Capsules* | |
| 25 | 721 |
| 26 | 249 |
| 27 | 233 |
| 28 | 258 |
| Mean | 365 |
| | P<0,03 |

TABLE 7 Experiment IV: Number of worms recovered when $L_3$ in suspension were either dosed *per os* or were injected into the rumen

| Group and Sheep No. | *T. falculatus* (No.) |
|---|---|
| *Per os* | |
| 21 | 1 581 |
| 22 | 976 |
| 23 | 466 |
| 24 | 1 007 |
| Mean | 1 008 |
| *Rumen* | |
| 29 | 77 |
| 30 | 396 |
| 31 | 732 |
| 32 | 122 |
| Mean | 332 |
| | P<0,03‡ |

‡ This can serve as an indication only, since the sheep were not allocated at random to the 2 groups

***Experiment IV.* A further comparison of gelatin capsules and suspension for administering *T. falculatus* $L_3$ *per os***

*Method*

Eight 3,5–8 month-old Dorper ewes were allocated at random to 2 groups (each of 4 sheep) dosed *per os* with $L_3$ either in suspension or in gelatin capsules. A further group of 4 wethers (6–18 months of age) was infested by injecting $L_3$ in suspension directly into the rumen.

A total of 7 344 $L_3$ of *T. falculatus* was divided into 3 doses, and administered daily over 3 days. Once again 100 % of the 200 $L_3$ examined were alive when examined after 38 months of cryopreservation.

*Results*

The worm burdens of the experimental animals are listed in Tables 6 & 7.

The $L_3$ administered in suspension developed significantly better than those administered in gelatin capsules or injected into the rumen (P<0,03). It must be mentioned, however, that only the comparison in Table 6 is, strictly speaking, valid, as the sheep used in the group injected into the rumen were not allocated at random to that group (Table 7).

*Comment*

Once again the $L_3$ in suspension, administered *per os*, developed significantly better (13,7 %) than those in gelatin capsules (5,0 %), administered by the same route (P<0,03), or those injected in suspension into the rumen. The results of this trial, however, were poorer than those with the same routes of infestation in the previous trials in the present series.

## DISCUSSION

It is gratifying that it appears from this series of trials that we have a practical method of infesting sheep with cryopreserved $L_3$ of *Trichostrongylus* spp. without having to resort to time-consuming laparotomy operations. We thus have a technique readily available for routine use in the laboratory.

Although relatively few animals were used per experimental group, it is obvious from this series of trials that cryopreserved $L_3$ of *Trichostrongylus* spp. in suspension develop better than when dosed in gelatin capsules.

One possible explanation for the comparatively poor results with $L_3$ in gelatin capsules is that the capsules end up in the rumen, and that $L_3$ in suspension usually bypass the rumen, whether or not the oesophageal groove reflex has been prestimulated with $CuSO_4$. This explanation is supported by the results of Dash (1981) who concluded that $L_3$ of *O. columbianum* in suspension usually bypass the rumen when dosed *per os*.

If this surmise is correct (and it can possibly be tested by including dextrose with the larval suspension—Hennessy & Prichard, 1979), then *per os* infestation with larvae in gelatin capsules will probably resemble more closely natural infestation (i.e. via the grazing) than *per os* administration of $L_3$ in suspension.

It is interesting that, with both the gelatin capsules (mean development of 5,0–13,1 %) and the suspension (13,7–34,9 %) development was markedly superior to that of previous trials (0,2 % with capsules and 2,7–4,3 % after injection of $L_3$ into the duodenum; Table 1).

Apart from the fact that large variations in viability occur between batches and individual vials of cryopreserved $L_3$ (Van Wyk *et al.*, 1977; Van Wyk & Gerber, 1980a), there is another possible explanation for this difference, namely the adaptation of the strain to cryopreservation by selection through serial passage, which, if true, may have far-reaching consequences.

While Van Wyk *et al.* (1977) and Van Wyk & Gerber (1980a) used strains of *T. falculatus* and *T. colubriformis* which had not previously been exposed to cryopreservation in the laboratory, the strain of *T. falculatus* used in the present experiments had been derived as follows: $L_3$ of the strain used by Van Wyk & Gerber (1980a) were thawed after cryopreservation and used to infest a donor sheep. $L_3$ isolated from the faeces of this sheep were used in the present series of experiments.

There were certainly differences in the percentage survival of the $L_3$ of *T. falculatus* on thawing, 91,3 % being recorded by Van Wyk *et al.* (1977) (their Table 14) and 94,1% by Van Wyk & Gerber (1980a) (their Table 1), compared to 100 % of 800 of these $L_3$ that were alive when thawed for the present series of experiments.

If certain species or strains of nematodes are changed by cryopreservation, the implications may be serious and should be known for routine use of this technique in the laboratory. The only investigation apparently thus far reported in this respect failed to show significant differences between cryopreserved and unfrozen *H. contortus* as regards susceptibility of a resistant strain to benzimidazole anthelmintics (Van Wyk & Gerber, 1980 b). This aspect requires further investigation.

### *M. marshalli*

It is clear from the results of Experiment II that only in very young, fully susceptible sheep did cryopreserved *M. marshalli* develop sufficiently well for routine propagation in the laboratory.

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