# A comparison of the infectivity of cryopreserved versus unfrozen infective larvae of *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Trichostrongylus axei*

## RESULTS OF THE ONDERSTEPOORT VETERINARY INSTITUTE AND COLLABORATORS FROM 1977 TO THE PRESENT

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**ABSTRACT**

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The infectivity for sheep of cryopreserved infective larvae (L3) of various strains of *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Trichostrongylus axei* is compared using previously published results of trials conducted at the Onderstepoort Veterinary Institute laboratories, and of collaborators.

The means and ranges of development were similar for both frozen and unfrozen larvae of two of the three worm species reviewed. A mean of 33,4 % (range, 12,7–63,0 %) of cryopreserved *H. contortus* L3 developed, compared to a mean of 43,7 % (range 2,4–78,7 %) of unfrozen L3 of this worm species. The corresponding values for *T. colubriformis* were 33,0 % (range 10,3–62,7 %), and 33,5 % (range 8,3–52,2 %), respectively. In the case of *T. axei*, the development of the cryopreserved L3 (tested in only three trials) was markedly lower than that of unfrozen L3 in the single trial in which the latter was evaluated.

It is concluded that development of cryopreserved L3 is probably similar to that of unfrozen L3 and that, for several reasons, maintaining nematode larvae in the frozen state in liquid nitrogen is a much superior method to that of one which entails cycling worm strains continually in their final hosts.

**Keywords:** Cryopreservation, cryopreserved vs unfrozen larvae, *Haemonchus contortus*, infective nematode larvae, infectivity, South Africa, *Trichostrongylus colubriformis*, *Trichostrongylus axei*

Recently, Conder & Johnson (1996) expressed the opinion that little has been done to assess the infectivity of gastrointestinal infective larvae (L3) exsheathed in sodium hypochlorite. They reached this conclusion after they had found that the L3 of several worm species exsheathed in sodium hypochlorite solution were poorly infective for gerbils (jirds—*Meriones unguiculatus*). Van Wyk (1998) commented that at least the following papers had reported relatively good infectivity of similarly exsheathed cryopreserved L3 of common gastrointestinal nematodes in sheep and cattle, after having been cryopreserved in liquid nitrogen for as long as 136 months: Campbell, Blair & Egerton (1972); Campbell & Thomson (1973); Kelly

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& Campbell (1974); Kelly, Campbell & Whitlock (1976); Van Wyk, Gerber & Van Aardt (1977); Van Wyk & Gerber (1980a); Rew & Campbell (1983); Van Wyk, Gerber & Alves (1984); Van Wyk, Gerber & Alves (1990b).

Subsequently, Van Wyk, Gerber & De Villiers (1999) reported a mean development of 22 % of L3 of nine ovine nematode species cryopreserved for 13,2–15,8 years, after the L3 had been exsheathed in sodium hypochlorite solution.

In the majority of South African trials undertaken to determine the longevity of L3 preserved by freezing in liquid nitrogen, the larvae were deposited at the predilection sites of the adult stages of the worm species concerned (Van Wyk *et al.* 1977; Van Wyk & Gerber, 1980a; Van Wyk *et al.* 1984; 1990b). The L3 of *H. contortus* frozen for lengthy periods, however, were also found to be viable when administered *per os* to sheep. In this respect Van Wyk & Gerber (1999?) found mean developments of 42 % and 66 % of two different batches of L3 of the Onderstepoort anthelmintic-susceptible strain of *H. contortus* after storing for almost 16 years in liquid nitrogen.

Kelly & Campbell (1974) and Van Wyk *et al.* (1977) have determined that the survival and infectivity of different batches of frozen larvae and even that of larvae of different vials of a given batch may be variable. The majority of the pure cultures of nematode species and strains used in the laboratories at Onderstepoort Veterinary Institute over the past 20 years have been maintained by exsheathing the L3 stages in sodium hypochlorite solution and subsequently freezing them in liquid nitrogen, generally with good viability when the L3 were thawed.

After numerous trials in South Africa involving both cryopreserved and unfrozen L3, the question arose as to whether the difference in the viability between the two categories of larvae warranted consideration, or if only that between different batches of cryopreserved L3 should be emphasized. The information in Tables 1 and 2 was gleaned from previously published South African papers originating from workers at the Onderstepoort Veterinary Institute and from collaborators, in order to review and compare the results obtained in sheep infected with either cryopreserved or unfrozen L3.

It will be noted in Table 1 that from a small number of infected animals more adult worms were recovered than the calculated number of L3 that they had received. This indicates that inaccuracies had occurred in the infective doses of L3, estimated from counts of aliquots of larval suspensions. The reason was probably because the aliquots examined in this laboratory are relatively small (J.A. van Wyk, unpublished observations 1999), as the principal aim in the trials has more been to administer similar infective dosages to the animals in a trial, than it has been accurately to estimate the actual numbers administered. Such cases of underestimation were, however, infrequent, and occurred in trials with both cryopreserved and unfrozen L3. Examples of the latter are apparent development of 101 %, 118 % and 109 % in individual sheep, as reported respectively by Van Wyk, Van Schalkwyk, Gerber, Visser, Alves & Van Schalkwyk (1989b, Table 4), Van Wyk, Malan, Gerber & Alves (1989a, Table 3) and van Wyk, Malan & Randles (1997b, Table 1).

From Table 1 it can be seen that a mean of 33,4 % ($n = 9$; range 12,7–63,0 %) of cryopreserved *H. contortus* L3 developed in sheep, compared with a mean of 43,7 % ($n = 18$; range, 2,4–78,7 %) for unfrozen L3 of the same species. The corresponding figures for *Trichostrongylus colubriformis* are 33,0 % ($n = 6$; 10,3–62,7 %) and 33,5 % ($n = 3$; 8,3–52,2 %). Cryopreserved *Trichostrongylus axei* developed relatively poorly ($n = 3$; 13,6 %, range 8,6–22,9 %), while 61,4 % of unfrozen L3 developed in the single trial conducted.

It should be kept in mind that in all these trials only batches of cryopreserved L3 were used that survived the freezing and thawing process well and not batches taken at random. Nevertheless, over the years fewer than 10 % of batches of L3 of the common ovine gastrointestinal nematodes did not freeze and thaw successfully by this method, as they mostly yielded in excess of 90 % of survivors that moved with alacrity once thawed (J.A. van Wyk, unpublished observations 1999). It appears from numerous observations that if the L3 in a batch are in good condition when thawed within a day or two of having been frozen in liquid nitrogen, they will survive well when frozen for much longer periods. This brings the practical possibility within a few days or weeks of freezing, to thaw some vials from each batch of L3 to evaluate the success of the freezing process. On the other hand, the batches of fresh, unfrozen L3 that are used for the maintenance of pure strains or in trials are also selected, only those with L3 moving with alacrity and almost 100 % alive being used (J.A. van Wyk, unpublished observations 1999).

In conclusion, it can unequivocally be stated that the L3 of most of the common worm species of sheep and cattle survive and remain infective for many years in the frozen state in liquid nitrogen. Furthermore, for the following reasons, cryopreservation is probably a superior method of maintaining nematode strains possessing particular biological characteristics, than is that of maintaining them by perpetually cycling them in animal hosts.

The lengthy survival of L3 in liquid nitrogen has the implication that the number of times that worm strains/species need to be cycled in their mammalian hosts is drastically reduced after their initial isolation. Thus the epidemiological characteristics of the strain are probably maintained with little change. For

TABLE 1 Comparison of the infectivity of cryopreserved and unfrozen gastrointestinal nematode larvae[a]

| Worm strain | Worm strain (months frozen) | Susc/ Res (S/R) | Infective larvae (L3) | | | | Worm development | | | Ref[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Trial (batch) + | % alive[b] | No. given | Route | Mean (%) | Min | Max | |
| *Haemonchus contortus:* cryopreserved ($n$ = 9) | | | | | | | | | | |
| 1. OP-Susc | < 1 | S | 1(a) | 53 | 276 | ABO | 12,7 | n/a | n/a | 1 |
| 1. OP-Susc | < 1 | S | 1(a) | 53 | 276 | DUO | 15,6 | n/a | n/a | 1 |
| 1. OP-Susc | 7 | S | 2(b) | 86 | 1 059 | ABO | 31,7 | 0 | 757 | 1 |
| 1. OP-Susc | 24 | S | 4(c) | 97 | 8 414 | ABO | 39,9 | 126 | 6 297 | 1 |
| 1. OP-Susc | 59 | S | 2(d) | 97 | 9 560 | ABO | 35,7 | 1 885 | 4 113 | 2 |
| 1. OP-Susc | 59 | S | 2(d) | 97 | 9 560 | /OS | 16,6 | 985 | 2 081 | 2 |
| 1. OP-Susc | 187 | S | 7(e) | 93 | 9 386 | /OS | 56,9 | 3 242 | 8 549 | 3 |
| 1. OP-Susc | 187 | S | 8(e) | 90 | 8 900 | /OS | 63,0 | 2 339 | 9 624 | 3 |
| 2. OP-TBZ | 159 | R | 9(a) | 88 | 4 820 | ABO | 28,5 | 285 | 2 417 | 4 |
| Mean: 33,4 % | | | | | | | | | | |
| *Haemonchus contortus:* Unfrozen ($n$ = 18) | | | | | | | | | | |
| 1. OP-Susc | nil | S | 3(b) | 100 | 1 059 | /OS | 29,6 | 133 | 704 | 1 |
| 1. OP-Susc | nil | S | 5(c) | 91 | 2 856 | /OS | 2,4 | 28 | 114 | 1 |
| 3. OP-M | nil | R | 6 | nd | 3 324 | /OS | 13,8 | 285 | 612 | 4 |
| 4. Cull-Unsel | nil | R | 7 | nd | 3 400 | /OS | 31,1 | 0 | 2 036 | 5 |
| 5. Cull-Sel | nil | R | 8 | nd | 3 120 | /OS | 23,3 | 33 | 2 277 | 5 |
| 6. PTZR | nil | R | 9 | nd | 3 009 | /OS | 43,3 | 830 | 1 880 | 6 |
| 7. Wh. River | nil | R | 10 | nd | 5 000 | /OS | 37,1 | 1 630 | 2 010 | 6 |
| 8. Sheepmoor | nil | R | 11 | nd | 3 000 | /OS | 78,7 | 1 477 | 3 538 | 7 |
| 9. Cullinan | nil | R | 12 | nd | 3 300 | /OS | 50,4 | 366 | 3 016 | 7 |
| 10. Wh. River | nil | R | 13 | nd | 5 000 | /OS | 54,8 | 943 | 3 990 | 7 |
| 11. Stellenb | nil | R | 14 | nd | 3 000 | /OS | 18,0 | 11 | 1 239 | 7 |
| 12. Swellend | nil | R | 15 | nd | 3 300 | /OS | 67,9 | 1 532 | 2 770 | 7 |
| 13. Hc Lev/Mor | nil | R | 16 | nd | 3 141 | /OS | 70,3 | 1 504 | 3 174 | 8 |
| 14. Veldram | nil | R | 17 | nd | 2 720 | /OS | 56,5 | 1 048 | 2 152 | 9 |
| 15. OP-Susc | nil | S | 18 | nd | 2 850 | /OS | 76,6 | 805 | 3 106 | 10 |
| 16. Howick | nil | R | 19 | nd | 3 150 | /OS | 62,5 | 631 | 2 605 | 11 |
| 17. Badplaas | nil | R | 20 | nd | 3 000 | /OS | 8,6 | 200 | 300 | 11 |
| 17. Badplaas | nil | R | 21 | nd | 3 000 | /OS | 62,2 | 1 020 | 2 639 | 11 |
| Mean: 43,7 % | | | | | | | | | | |
| *Trichostrongylus colubriformis:* Cryopreserved ($n$ = 6) | | | | | | | | | | |
| 1. OP-Susc | < 1 | S | 1(a) | 96 | 385 | ABO | 10,3 | n/a | n/a | 1 |
| 1. OP-Susc | < 1 | S | 1(a) | 96 | 385 | DUO | 10,6 | n/a | n/a | 1 |
| 1. OP-Susc | 8 | S | 2(a) | 100 | 712 | DUO | 45,5 | 86 | 507 | 1 |
| 1. OP-Susc | 24 | S | 3(a) | 94 | 1 355 | DUO | 62,7 | 553 | 926 | 1 |
| 1. OP-Susc | 59 | S | 4(a) | 96 | 967 | DUO | 37,7 | 269 | 429 | 1 |
| 1. OP-Susc | 166 | S | 5(a) | 68 | 9 690 | DUO | 31,1 | 2 645 | 3 547 | 4 |
| Mean: 33,0 % | | | | | | | | | | |
| *Trichostrongylus colubriformis:* Unfrozen | | | | | | | | | | |
| 1. OP-Susc | nil | S | 2(b) | 99 | 803 | /OS | 52,2 | 336 | 629 | 1 |
| 1. OP-Susc | nil | S | 3(c) | 94 | 1 991 | /OS | 8,3 | 78 | 301 | 1 |
| 2. Nott. Rd | nil | R | 6 | nd | 4 800 | /OS | 40,1 | 1 223 | 2 649 | 12 |
| Mean: 33,5 % | | | | | | | | | | |
| *Trichostrongylus axei:* Cryopreserved ($n$ = 3) | | | | | | | | | | |
| 1. OP-Susc | 8 | S | 2(a) | 98 | 1 021 | ABO | 9,4 | 0 | 253 | 1 |
| 1. OP-Susc | 59 | S | 3(a) | 98 | 13 322 | ABO | 22,9 | 1 778 | 6 704 | 2 |
| 1. OP-Susc | 59 | S | 3(b) | 98 | 13 322 | /OS | 8,6 | 263 | 1 815 | 2 |
| Mean: 13,6 % | | | | | | | | | | |
| *Trichostrongylus axei:* Unfrozen ($n$ = 1) | | | | | | | | | | |
| 1. OP-Susc | nil | S | 2(b) | 100 | 845 | /OS | 61,4 | 138 | 630 | 1 |

[a] Abbreviations: n/a – not applicable; ABO – abomasum; DUO – duodenum; /OS – per os; S – anthelmintic-susceptible; R – anthelmintic-resistant; Min – minimum; Max – maximum; Ref – reference; Susc/Res – susceptible/resistant; m – months

[b] nd – not determined, but on inspection the larvae were judged to be practically 100 % alive

[c] See Table 2 for complete references, and the full names of the various worm strains

\+ The trial and batch numbers indicate where L3 species were used in the same trials, and where the same batches were used in different trials

TABLE 2 Worm strains reviewed: References[a]

| Worm species and strain | Susc/Res (S/R) | Reference[b] |
|---|---|---|
| *Haemonchus contortus:* Cryopreserved | | |
| 1. OP-susceptible | S | 1. Van Wyk, Gerber & Van Aardt (1977) |
| 1. OP-susceptible | S | 2. Van Wyk & Gerber (1980a) |
| 1. OP-susceptible | S | 3. Van Wyk & Gerber (1999—submitted for publication) |
| 2. OP-TBZ | R | 4. Van Wyk, Gerber & De Villiers (1999) |
| *Haemonchus contortus:* Unfrozen | | |
| 3. OP-MBZ | R | 4. Van Wyk, Gerber & De Villiers (1999) |
| 4. Cullinan/unselected | R | 5. Van Wyk, Gerber & Alves (1982) |
| 5. Cullinan/selected | R | 5. Van Wyk, Gerber & Alves (1982) |
| 6. PTZR | R | 6. Van Wyk, Malan, Gerber & Alves (1987) |
| 7. White River | R | 6. Van Wyk, Malan, Gerber & Alves (1987) |
| 8. Sheepmoor | R | 7. Van Wyk, Malan, Gerber & Alves (1989a) |
| 9. Cullinan | R | 7. Van Wyk, Malan, Gerber & Alves (1989a) |
| 10. White River | R | 7. Van Wyk, Malan, Gerber & Alves (1989a) |
| 11. Stellenbosch | R | 7. Van Wyk, Malan, Gerber & Alves (1989a) |
| 12. Swellendam | R | 7. Van Wyk, Malan, Gerber & Alves (1989a) |
| 13. Hc Lev/Mor | R | 8. Van Wyk, Van Schalkwyk, Gerber, Visser, Alves & Van Schalkwyk (1989b) |
| 14. Veldram | R | 9. Van Wyk, Van Schalkwyk, Bath, Gerber & Alves (1991) |
| 15. OP-susceptible | S | 10. Van Wyk, Malan, Van Rensburg, Oberem & Allan (1997a) |
| 16. Howick | R | 11. Van Wyk, Malan & Randles (1997b) |
| 17. Badplaas | R | 11. Van Wyk, Malan & Randles (1997b) |
| *Trichostrongylus colubriformis:* Cryopreserved | | |
| 1. OP-susceptible | S | 1. Van Wyk, Gerber & Van Aardt (1977) |
| 1. OP-susceptible | S | 2. Van Wyk and Gerber (1980a) |
| 1. OP-susceptible | S | 4. Van Wyk, Gerber & De Villiers (1999) |
| *Trichostrongylus colubriformis:* Unfrozen | | |
| 2. Nottingham Road | R | 12. Van Wyk, Bath, Gerber & Alves (1990a) |
| *Trichostrongylus axei:* Cryopreserved | | |
| 1. OP-susceptible | S | 2. Van Wyk & Gerber (1980a) |
| *Trichostrongylus axei:* Unfrozen | | |
| 1. OP-susceptible | S | 1. Van Wyk, Gerber & Van Aardt (1977) |

[a] See Table 1 for abbreviations. Note that abbreviations used in the names of the worm strains, refer either to the origins, or specific characteristics, both of which have no relevance to the present paper

[b] The numbers of the references correspond with those in Table 1

instance, Van Wyk *et al.* (1977; 1990b) detected no tendency for the cryopreserved L3 of *H. contortus* to become hypobiotic, although Hendrikx, Boersema & Eysker (1988) considered that cryopreservation might have reduced the level of hypobiosis in a strain of *H. contortus* conditioned for inhibited development.

The high percentages of L3 which survive cryopreservation (Table 2) imply that a smaller selection pressure is placed on L3 stored in liquid nitrogen, than is undergone by those subjected to repeated cycles of infection and larval recovery. In other words, it appears that the original genetic variability of the former is preserved practically unchanged, particularly because the infectivity of the cryopreserved larvae is apparently not compromised, as suggested by the comparisons of the infectivity of cryopreserved and unfrozen larvae in Table 1.

The low temperature of liquid nitrogen protects L3 from destruction by fungi, as often happens with L3 stored at room temperature or in a refrigerator.

Since few, if any, worm strains can be distinguished morphologically from one another, repeated cycling of a worm strain increases its chances of cross-contamination with one or more strains of the same species, without it being detected. In this way valuable strains may be lost.

Exsheathed cryopreserved L3 of at least some worm species survive after thawing for a sufficiently long period for them (like L3 that have not been frozen), to be despatched worldwide without losing their infectivity. An example of this is the L3 of a strain of *H. contortus* which was successfully established in sheep despite the fact that they had been delayed after airfreighting, in the customs department of an airport for more than a week (J.A. van Wyk, unpublished observations 1999).

Van Wyk & Gerber (1980b) found no significant effect on the anthelmintic susceptibility status of a benzimidazole-resistant strain of *H. contortus* cycled five times in series through sheep, with freezing and thawing treatments in each cycle, compared to another subculture of the same strain cycled similarly, but without cryopreservation.

By cryopreserving L3 in liquid nitrogen, the costs of maintaining worm strains are cut drastically, when compared to those, which involve maintenance in donor animals.

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