RESEARCH COMMUNICATION

Oral infectivity of cryopreserved *Haemonchus contortus* infective larvae that recovered relatively slowly when thawed after more than 15 years in liquid nitrogen

J.A. VAN WYK[1] and H.M. GERBER[2]

ABSTRACT

VAN WYK, J.A. & GERBER, H.M. 2000. Oral infectivity of cryopreserved *Haemonchus contortus* infective larvae that recovered relatively slowly when thawed after more than 15 years in liquid nitrogen. *Onderstepoort Journal of Veterinary Research*, 67:149–152

Infective larvae (L3) of two different batches of anthelmintic-susceptible *Haemonchus contortus*, cryopreserved for 15,6 and 15,7 years in liquid nitrogen appeared dead when examined 10–20 min after thawing. However, having become motile later in the day, their viability was tested the next day in two separate groups of sheep.

The mean development of 41,8 % and 66,3 % of the *H. contortus* compared favourably with previous trials with this strain, in which the L3 were injected into the abomasa of sheep after various periods of cryopreservation.

It is speculated that the relatively slower recovery rates after thawing of the L3 used in this trial, when compared with those in previous trials in which one of the two batches of susceptible *H. contortus* was used after shorter periods of cryopreservation, may be an indication that these L3 were gradually approaching the limits of survival after almost 16 years in liquid nitrogen.

Keywords: Cryopreservation, *Haemonchus contortus*, larva longevity, oral infection

INTRODUCTION

Cryopreservation of gastrointestinal nematode infective third-stage larvae (L3) after exsheathing in sodium hypochlorite solution has become the mainstay for maintaining various strains of these parasites in our laboratory, but it is imperative to know for how long the L3 can be stored before having to recycle them through their vertebrate hosts.

Van Wyk, Gerber & De Villiers (2000) reported on the development of several species of gastrointestinal nematode infective larvae (L3) when administered parenterally to sheep after 13,3–15,8 years of cryopreservation. Their report described the results obtained in a series of trials, in which batches of the L3 of nematode species parasitising sheep and/or cattle were cryopreserved and some of the vials containing them thawed at irregular intervals in order to assess their longevity and infectivity by depositing them parenterally at their predilection sites in their respective hosts.

At the time of preparing for the above trials, it was noticed that practically all the L3 of two different batches of the Onderstepoort anthelmintic-susceptible strains (the OP-susc[a] and OP-susc[b]) of *Haemonchus contortus* cryopreserved for 15,6 and 15,7 years, respectively, appeared to be dead when examined

[1] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa, and ARC-Onderstepoort Veterinary Institute, Onderstepoort, 0110 South Africa

E-mail: janvw@moon.ovi.ac.za

[2] Deceased

Accepted for publication 27 January 2000—Editor

within 10–20 min of being thawed and suspended in 0,09 % saline solution. Usually, the vast majority of *H. contortus* L3 are very active after this period of time. The thawed L3 were placed aside with the intention of discarding them, but when re-examined after about 10 h, many of them appeared to be alive. It was decided to determine whether they could still infect sheep.

Previously, Rew & Campbell (1983) reported development of 49–68 % of L3 of *H. contortus,* that were motile when thawed after 10 years of cryopreservation and administered orally to sheep.

MATERIALS AND METHODS

Cryopreservation of the *H. contortus* L3

H. contortus L3 were obtained by making faecal cultures as described by Reinecke (1973) and were exsheathed in sodium hypochlorite 0,09 % sodium chloride solution. Thereafter they were washed on wet- strengthened filter paper, concentrated in a 0,09 % NaCl solution, and frozen in the gas phase of liquid nitrogen (Van Wyk, Gerber & Van Aardt 1977; Van Wyk & Gerber 1980). The vials were maintained either in the gas phase of, or submerged in, the liquid nitrogen.

To thaw the larval suspensions, the vials were gently shaken in water at 55 °C until only a small pellet of ice remained in each vial, whereafter the vials were immediately whirled in cold tap water at approximately 25 °C to ensure that the contents were not exposed to excessive heat. The larval suspensions were diluted with 0,09 % saline solution in measuring cylinders and the numbers of living and dead larvae in each suspension were estimated after a period of approximately 10–15 min, by examining 100 L3 after a few drops of 10 % formalin solution had been added to each aliquot of suspension to stimulate movement.

The two batches of larvae mentioned above, were re-examined when they had been at room temperature for about 30 h after thawing. One hundred L3 of each batch were examined using a stereo microscope and another 100 with a standard microscope to establish the proportions of larvae that had survived the cryopreservation. Before microscopical examination a drop of 10 % formalin was added to each aliquot of larval suspension to stimulate larval movement. Those which did not move while being inspected microscopically were considered to be dead.

Experimental animals

Six yearling Dorper sheep were used in the trial. They had been born and raised under conditions of minimal exposure to gastrointestinal nematodes and, for the duration of the trial, were fed sterilized hay and a pelleted concentrate ration. They were maintained in concrete-floored pens, which were thoroughly swept on 5 days per week.

Faecal worm egg counts

Faecal examinations by a modified McMaster method (Reinecke 1973), and by the flotation method of Whitlock (1959), using 5 g of faeces in each case, did not reveal the presence of worm eggs in the animals on the day after the sheep had been infected with the thawed L3, thus indicating that (as far as we could ascertain) the sheep were practically worm-free when they were infected for the trial.

Infection of the sheep

After the concentrations of live *H. contortus* L3 in the two batches had been estimated, three sheep per batch were each infected *per os* with an estimated 2 000 live larvae suspended in 0,09 % sodium chloride solution.

Worm recovery and identification

The sheep were slaughtered for worm recovery 35 days after infection. Their abomasal mucosae were digested, and the digests and abomasal contents from each sheep concentrated separately on 38 µm sieves, as described by Reinecke (1973). If more than 50 worms were present in an aliquot of a suspension of abomasal ingesta, the first 50 recovered were identified by examination under a standard light microscope. If, however, less than this number were present, all were identified.

RESULTS

About 10 h after thawing, 93 % of the OP-susc[a] batch of anthelmintic-susceptible *H. contortus* L3 were alive (Tables 1 and 2), but this proportion was not

TABLE 1 Particulars of worm strains and batches of infective third-stage larvae (L3) of *Haemonchus contortus* examined

Worm species or strain[a]	Concentration of larvae/mℓ	Survival of *H. contortus* infective larvae (%)		
		2 h	10 h	30 h
H.c (OP-susc[a])[b]	38 000	nd[c]	93	93
H.c (OP-susc[b])	61 000	nd	nd	90

[a] H.c (OP-susc): *Haemonchus contortus* (OP-susceptible) strain
[b] Two vials of L3 of this batch of *H. contortus*, which was used in previous trials by Van Wyk *et al.* (1977; 1990) and Van Wyk & Gerber (1980), were thawed for gauging the longevity of this species in liquid nitrogen
[c] nd: not determined

TABLE 2 Worm development of the two batches of the Onderstepoort susceptible strain of *H. contortus* used in the trial

Group of sheep[a]	Strain of *H. contortus*	Years frozen	Infective larvae (L3)[b]	
			Survival	Viability
1	OP-susc[a]	15,6	93,0	41,8 (34,5–45,0)
2	OP-susc[b]	15,7	89,5	66,3 (26,2–107,8)

[a] 3 sheep per group
[b] Mean percentage (range) of survival and development (viability) of the L3 drenched

determined for the larvae in the OP-susc[b] batch until the next day. After a total of about 30 h, 93% and 90% were found to be alive, respectively, in the OP-susc[a] and OP-susc[b] batches of *H. contortus*, and the mean percentages of development of the respective larval batches were 41,8% and 66,3%.

DISCUSSION

These results, together with those of Rew & Campbell (1983) and Van Wyk *et al.* (1999) show conclusively that at least some batches of cryopreserved *H. contortus* L3 have the potential to develop very effectively by oral infection, even after 10–15 years.

Probably the best lesson to be learnt from the results of this trial is that L3 that appear to be lethargic soon after having been thawed, are not necessarily poorly infective; they should be left for some hours before a final decision concerning their condition is made. On the other hand, it is important to heed the warning of Van Wyk, Gerber & Alves (1990) that, even though few batches do not survive cryopreservation well (Van Wyk *et al.* 1999) the L3 used in this trial and those of Van Wyk *et al.* (1977; 1990; 2000) and Van Wyk & Gerber (1980) were from selected batches. In other words, batches, in which practically all of the L3 did not move with alacrity when thawed, were not considered for use in the trials. Furthermore, as can be expected, not every batch of L3 appearing lethargic immediately after thawing is viable!

The results further support the contention of Van Wyk (1998) that the suggestion of Conder & Johnson (1996) that sodium hypochlorite caused L3 of *H. contortus, Ostertagia ostertagi* and *Trichostrongylus colubriformis* to be poorly viable in the gerbil (jird, *Meriones unguiculatus*) in their trials, was incorrect.

After cryopreservation for almost 16 years, the larvae of the OP-susc[a] batch were at least as infective to sheep as were others that had been preserved similarly for shorter periods (Van Wyk *et al.* 1977, 1990, 2000; Van Wyk & Gerber 1980). This was quite unexpected as, not only had the larvae appeared dead soon after thawing, but they had been administered *per os* after a lapse of 30 h between thawing and infection, whereas in the previous trials they were administered by injection directly into the lumen of the abomasum about 6 h after thawing.

As has also occurred in a few previous trials in this laboratory (Van Wyk 1999), the percentage development of the Onderstepoort anthelmintic-susceptible strain of *H. contortus* in one of the sheep in the OP-susc[a] batch was higher than the estimated number of L3 administered, indicating overestimation of the number of L3 drenched to this sheep. The infective dose was calculated from the estimated percentage of live L3, and it is possible that some of the larvae which did not move when inspected microscopically were considered as dead but, in fact, were alive.

After short periods of cryopreservation of a few months' duration in liquid nitrogen, *H. contortus* L3 usually recover quickly after thawing, becoming practically fully active within 10–20 min. In the trials reported by Van Wyk & Gerber in 1980 and Van Wyk *et al.* in 1990 the percent survival was only estimated after at least 8 h (thus after the trial sheep had been infected). For this reason the results obtained cannot be compared directly with the present ones; in the earlier trials the L3 were also only cursorily examined shortly after thawing, at which stage they seemed to be in good condition (J.A. van Wyk, unpublished observations 1978; 1988). Nevertheless, the fact that the larvae definitely recovered more slowly after having been thawed in the present trial, than larvae frozen for much shorter periods, may perhaps be an indication that the time-span of 16 years of cryopreservation is gradually approaching the limit of survival of L3 of *H. contortus* in liquid nitrogen.

The development of the *H. contortus* L3 in the present trials was not much different to those used by Rew & Campbell (1983), who reported that 67% of *H. contortus* L3 cryopreserved for 10 years were motile when thawed. Unfortunately they do not mention how soon after thawing the L3 were examined. However, in the light of the 90 and 93% survival rates in the present trial, it seems likely that they estimated the percentage soon after thawing, particularly considering the similar percentages of development of the L3 in their trial and the present one (49–68%, versus 42–66%, respectively).

It is unfortunate that no more batches of cryopreserved OP-susc[a] L3 of *H. contortus* remain, which may have enabled the end point of their longevity after storage in liquid nitrogen to be determined.

ACKNOWLEDGEMENTS

Prof. Roy Tustin is thanked for a valued contribution towards improving this article.

REFERENCES

CONDER, G.A. & JOHNSON, S.S. 1996. Viability of infective larvae of *Haemonchus contortus*, *Ostertagia ostertagi*, and *Trichostrongylus colubriformis* following exsheathment by various methods. *Journal of Parasitology*, 82:100–102.

REINECKE, R.K. 1973. *The larval anthelmintic test in ruminants.* (Technical Communication, no. 106, Department of Agricultural Technical Services, Republic of South Africa).

REW, R.S. & CAMPBELL, W.C. 1983. Infectivity of *Haemonchus contortus* in sheep after freezing for ten years over liquid nitrogen. *Journal of Parasitology,* 69:251–252.

VAN WYK, J.A. 1998. Viability of nematode larvae after exsheathment with sodium hypochlorite. *Parasitology Today,* 14:474–475.

VAN WYK, J.A. 1999. 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. *Onderstepoort Journal of Veterinary Research,* 66:285–289.

VAN WYK, J.A. & GERBER, H.M. 1980. Survival and development of larvae of the common nematodes of ruminants after long-term cryopreservation and investigation of different routes of infestation. *Onderstepoort Journal of Veterinary Research,* 47:129–136.

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.

VAN WYK, J.A., GERBER, H.M. & DE VILLIERS, F.J. 2000. Parenterally administered gastrointestinal nematode infective larvae viable after more than 15 years in liquid nitrogen. *Veterinary Parasitology*, 88:239–247.

VAN WYK, J.A., GERBER, H.M. & VAN AARDT, W.P. 1977. Cryopreservation of the infective larvae of the common nematodes of ruminants. *Onderstepoort Journal of Veterinary Research,* 44:173–194.

WHITLOCK, H.V. 1959. The recovery and identification of the first stage larvae of sheep nematodes. *Australian Veterinary Journal,* 35:310–316.