Lungworm in Red Deer (Cervus elaphus) in New Zealand: Dictyocauslus viviparus or D. eckerti?

M Johnson, R Labes, M Taylor and C Mackintosh

Abstract

In the absence of evidence to the contrary, the lungworm infecting red deer in New Zealand has been assumed to be *Dictyocaulus viviparus* This paper presents molecular, morphological and cross-infective challenge evidence that it is *D eckerti*

Lungworm were collected from farmed red deer that had had no known cattle contact and from dairy cattle that had had no known deer contact Preliminary molecular studies, a comparison of the ITS-2 sequence of the lungworm derived from red deer to sequences deposited in the Genbank database (2000), indicated that the lungworm derived from red deer were *D. eckerti* Scanning electron microscopy of the mouthparts of lungworm of either red deer or cattle origin provided evidence of morphological differences, the mouthparts of lungworm from red deer matching a previously published description for *D eckerti*

A cross-infection study showed that the deer lungworm differed from cattle lungworm with respect to survival and patency in both deer and cattle These results provide strong evidence that the lungworm normally infecting farmed red deer in New Zealand is *D eckerti* and not *D. viviparus.*

Introduction

Since the establishment of the genus *Dictyocaulus* by Railliet and Henri in 1907 there has been disagreement among workers as to the number of species within it

The type species, *D. filaria*, the lungworm of sheep and goats has never been disputed, nor has the lungworm of horses and donkeys, *D arnfieldi Dictyocaulus viviparus* is universally acknowledged to occur in cattle However, morphologically it is very similar to *D eckerti*, the lungworm first described from reindeer by Skrjabin in 1931

During the period 1941–1988 the existence of *D eckerti* as a species was hotly disputed in the literature (Dougherty, 1946, Boev, 1957, Swietlikowski, 1961, Hugonnet et al , 1980) Gibbons and Khalil (1988) reviewed the genus on a morphological basis and acknowledged the existence of *D. eckerti* as a separate species Epe *et al* (1995) compared DNA from lungworms isolated from fallow deer, cattle, sheep and horses using a random amplified polymorphic DNA polymerase chain reaction technique (RAPD-PCR). All four lungworm isolates produced characteristic individual banding patterns and were assumed to be separate species Other authors, using various molecular methods, including the comparison of the second internally transcribed spacer sequences (ITS-2), (Epe *et al.*, 1997, Hoglund *et al*, 1999, Divina *et al*, 2000) have also concluded that *D eckerti* is indeed a species in its own right, but none have examined the lungworm of red deer

The series of investigations described below provide strong evidence that the species of lungworm found in farmed red deer in New Zealand is *D eckerti*

In the literature there are references to attempts to infect either cattle with lungworm of deer origin or deer with D *viviparus* but the results are conflicting and confusing A trial was conducted to investigate whether cross- infection with *D viviparus* and *D eckerti* can occur between red deer and cattle

Materials and Methods

Speciation

Scanning electron microscopy (SEM)

Eight lungworm derived from cattle and eight lungworm derived from deer were prepared for SEM in the standard way, sputter coated with gold and mounted *en face* They were examined using a Cambridge Stereo scan 360 scanning electron microscope.

The mouthparts of the two samples of lungworm were compared Both vertical and horizontal measurements were made of the oral opening, each measurement being recorded on an individual photograph

A ratio was then calculated (horizontal / vertical) to determine whether the oral opening was circular (*D. viviparus*) or elongate (*D eckerti*)

Molecular speciation

Cattle-derived lungworm were isolated from calves on a dairy farm on the Taieri plain The farm was well established and there was no record of deer having been seen on the pastures

Deer-derived lungworm were isolated from deer at AgResearch Invermay No cattle have grazed the paddocks from which the lungworm were isolated

Genomic DNA was extracted from four lungworm from each source (Johnson *et al*, in press) The extracted DNA was then sequenced in both directions

A BLAST (Basic local alignment search tool) search was performed against all entries in the Genbank database (2000)

Cross infection trial

Twelve dairy calves and twelve red deer fawns (*Cervus elaphus*) were hand-reared indoors from three-days and one-day old, respectively The animals were maintained in parasite- free conditions in a designated building. They were regularly monitored for both gastro-intestinal parasites and lungworm

Cattle and deer origin lungworm were sourced as given in the molecular speciation section The first stage larvae (L1) were extracted from the faeces using a modified Baermans technique The larvae, in approximately 300mls of water, were then placed in conical flasks held at room temperature and gently aerated After six days the larval suspension was poured into a large test tube and held at 4°C overnight to sediment down The supernatant was drawn off the top of the tube leaving the third stage larvae (L3s) in the bottom The L3s were placed in a culture flask and stored at 4°C

Three days prior to infection day the larvae of each species were pooled into weekly culture batches. There were only a limited number of cattle origin larvae available so to make a dose of 700 L3 per animal, L3s had to be drawn from a culture period spanning 3 weeks. The deer-origin larvae were matched so that there were no differences in the age of culture either between species or between host animals

The flasks were returned to storage at 4°C Twelve hours prior to infection the flasks were removed and left at room temperature for the larvae to warm up Just prior to infection the flasks were checked for larval motility

The calves and fawns, aged between three and four months, were infected with L3s as shown in Table 1 The larval dose was poured straight from the culture flask down the animals' throats The health of the animals was closely monitored through out the trial, observations of respiration and temperatures being taken daily

Table 1 Allocation of parasites and experimental hosts

Experimental host (n = 6)	Lungworm origin (dose = 700 L3)
Red Deer	Red Deer
Red Deer	Cattle
Cattle	Cattle
Cattle	Red Deer

The animals were faecal sampled at day 0, day 7, day 14 and daily from day 19 to day 3 post-infection to ascertain the presence of L1s

The animals were slaughtered and the lungs removed at day 35 post infection The lungs were then opened and the numbers of adult lungworm present counted

Results

Speciation

SEM

The mean ratio of the mouthparts was 1 27 for cattle lungworm and 1 85 for deer lungworm Analysis of variance showed the two ratios to belong to separate populations (SED= 0 089,p<0 001) There was no overlap in the measurements

The differences in the shape of the oral opening in both species are shown in Figure 1

D viviparous *D eckerti*

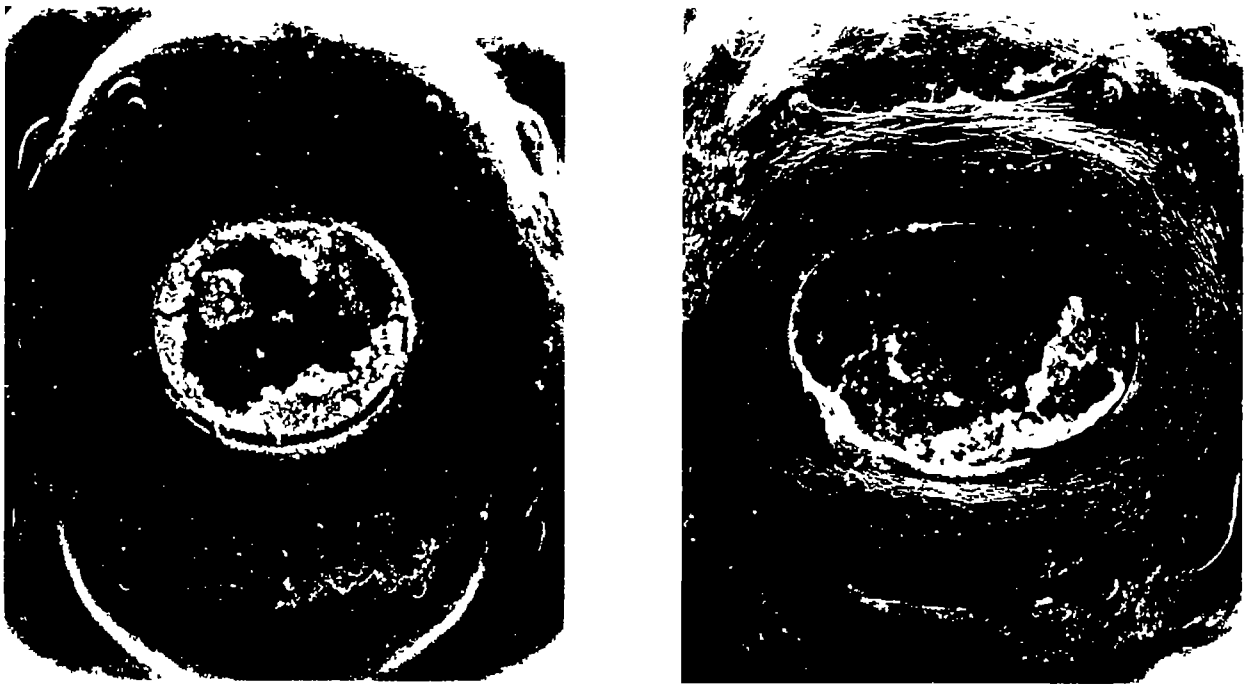

Figure 1. Scanning electron micrographs of the mouths of lungworms derived from cattle (*D viviparus* x 913) and deer (*D eckerti* x 1070)

Molecular speciation

The sequence obtained for lungworm derived from cattle matched that deposited for *D viviparus* ITS-2 with a BLAST score of 825 an E value of 0 0 and identity of 97% The next best match was with *D eckerti* ITS-2 with a BLAST score of 123 an E value of 1e-25 and identity of 84%

The sequence obtained for lungworm derived from red deer matched that deposited for *D eckerti* ITS-2 with a BLAST score of 347 an E value of 2e-93 and identity of 94% The next best match was with *D viviparus* ITS-2 with a BLAST score of 89 7 an E value of 8e-16 and identity of 83%

Cross infection trial

All the infections became patent in all the groups However, the cattle infected with *D eckerti* were only patent at low levels for a short period. The pre-patent period was 22 days in deer and 23 days in cattle

The pattern of LI production differed in each host parasite combination See Figure 2

Analysis of variance showed the differences between larval outputs to be significantly different (SED 0 803 p<0 001)

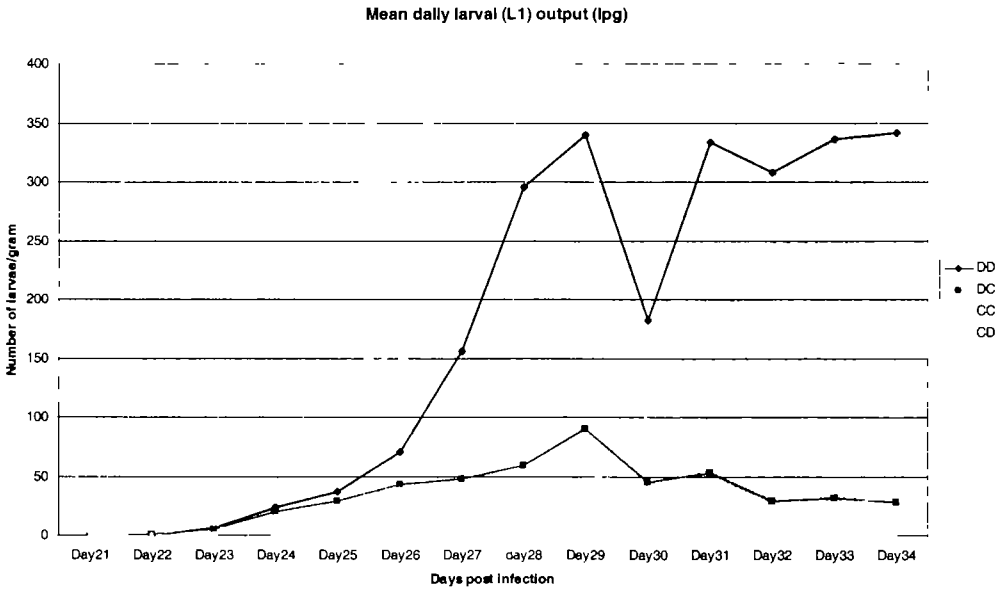

Figure 2. Mean daily larval (L1) output by host measured as larvae per gram of faeces

The numbers of adults remaining in the lung at day 35 post infection also varied significantly with each host parasite combination (Figure 3) The significance of this data was tested by randomisation The result obtained was not regenerated in a 10,000 times analysis

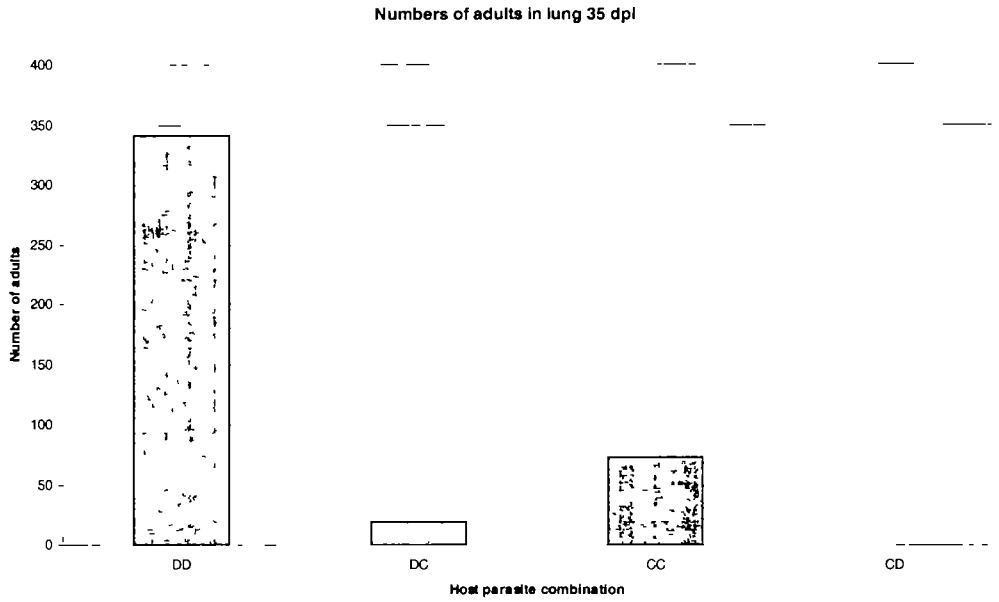

Figure 3. Mean number of adult lungworm found in host lungs at day 35-post infection

Discussion

From the molecular and morphological evidence presented it was concluded that the lungworm infecting farmed red deer in New Zealand is indeed *D eckerti*

Although not described above, an attempt was made to speciate the worms using light microscopy Linda Gibbons, a United Kingdom taxonomy expert, provided the protocol for the preparation and mounting of the worms The exercise showed why there has been so much debate over the years Using light microscopy it is indeed difficult to categorically split the worms into separate groups, as the gross morphological differences between them are small and measurements using light microscopy lack sensitivity This problem was noted by Divina *et al.* (2000) ' it appeared that the BCW thickness and length of lungworms exhibited wide variation '

Even Swietlikowski (1961), not a proponent of the existence of *D eckerti,* concluded "Studies on the systematics and taxonomy of the genus *Dictyocaulus* cannot be based mainly on the morphological examinations of adult specimens as the case was till now, the data on these worms biology are necessary too, to serve as a foundation to such studies and particularly so the data on biological adaptation to the hosts"

SEM provided a much more sensitive tool for measurement and confirmed the opinions of authors such as Jansen and Borgesteede (1990) and Gibbons and Khalil (1988) who concluded that, among other morphological differences such as cuticular ridges and spicule length, the shape of the oral opening was indeed different in the two species

The second internally transcribed spacer unit (ITS-2) is situated between two genes coding for ribosomal RNA, the 5 8s and 28s These genes are highly conserved across many species of nematode and are thus ideal for the design of universal primers that can be used to amplify the spacer DNA between them Many recent studies have demonstrated that the ITS-1 and ITS-2 regions provide accurate species markers for a range of bursate nematodes (Gasser and Hoste, 1995, Gasser *et al*,

1996, Epe *et al*, 1997, Hoglund *et al*, 1999) Our results confirmed that the ITS-2 sequence of D *viviparus* in New Zealand cattle matches that deposited in the Genbank database for *D. viviparus* for European cattle The E value for our sequence match was zero The E or expectation value is an indication of the number of possible alignments with a BLAST score equivalent to or greater than that achieved for the match in question that might occur by chance in a data base search The more negative the E value, the more significant the BLAST score. The best match for the sequence for lungworm derived from red deer was *D eckerti* with a lower E value and a higher BLAST score than the next best match which was *D viviparus* The BLAST score indicates the degree of sequence alignment, the higher the score the greater the alignment

In the literature it is possible to find references to cross infection experiments where cattle have been infected with larvae gathered from either wild or captive deer There are also experiments where deer have been infected with larvae from cattle All produce conflicting results, (Enigk and Hildebrandt ,1965, Presidente and Knapp, 1973, Corrigall *et al*, 1980, Foryet *et al*,2000), but none, with the exception of that of Bienioschek *et al* (1996) have been particularly uniform or well controlled In many trials the animals were of different ages, were not parasite naive, were already infected with lungworm when dosed for the experiment, the dose rates differed across the experiment and in many cases the dose rates were excessive

The results of this trial provided further evidence for the existence of two species of lungworm, echoing Swietlikowski's comment on the necessity for data on the biology of the worms in a speciation decision

All host parasite combinations became patent but the patterns of larval output varied. It is unfortunately not possible to directly compare the levels of larval output, measured in larvae per gram (lpg) of faeces, between deer and cattle with the data provided here as the total amount of faeces excreted daily is very different Larval counts are estimated from a daily 10-gram sample. Deer infected with *D. eckerti* produced more larvae per gram on a daily basis than did those infected with *D viviparus* Larval production climbed quickly in *D eckerti* infected deer and remained steady until the end of the trial This would indicate a well-established population of parasites, whereas larval production by *D. viviparus* infected deer peaked at 29 dpi (at a lower level than *D. eckerti*) and then began to decrease. This might suggest that the parasitic infection was being resolved by the host Only a small number of *D eckerti* L1s were found in the cattle faeces over a period of two days whereas *D. viviparus* production continued throughout the trial, steadily increasing and then levelling off. The pattern of larval production in the specific hosts was similar, with an increase and then maintenance, whereas in the non-specific host, larval production began to decrease well before the end of the trial. When the lungs were dissected at the end of the trial there were more adult *D eckerti* than *D. viviparus* in the deer lung. Almost half the original dose of *D. eckerti* L3s, on average, matured and survived in the deer lungs until the trial was terminated Only 2% of the original *D viviparus* dose was present No *D eckerti* were recorded in the cattle lungs at the end of the trial but approximately 10% of the original *D viviparus* dose was found

When the lungs were examined prior to dissection the cattle lungs that had been infected with *D viviparus* showed signs of oedema, emphysema and tissue damage whereas those that had been infected with *D eckerti* appeared normal There were species-related differences in the deer lungs Those infected with *D viviparus* exhibited more damage than those infected with *D eckerti* but the differences were not as marked as in the cattle lungs This might suggest that each species provokes a different response in each host or perhaps a differing degree of response

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