THE EFFECTS OF CONCURRENT EXPERIMENTAL INFECTIONS OF SHEEP WITH Trichostrongylus colubriformis AND T. vitrinus ON NEMATODE DISTRIBUTIONS, NUMBERS AND ON PATHOLOGICAL CHANGES

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Summary:
Simultaneous infections of Trichostrongylus colubriformis and T. vitrinus in the small intestine of the sheep were examined by comparing the numbers of worms which established and their distribution within the intestine in both monospecific infections and mixed infections. The results differed depending upon the species and number of parasites. The establishment of T. colubriformis was reduced and the distribution of the nematode population was displaced posteriorly within the intestine when 30,000 larvae of both species were administered, compared with pure infections of T. colubriformis. The reduced establishment was less marked with infections of 15,000 larvae of both species and there was only a slight posterior displacement of T. colubriformis. Neither effect was evident with infections of 7,500 larvae of both species. The rate of establishment and distribution of T. vitrinus were unaffected by the presence of T. colubriformis at all three rates of infection.

Atrophy of villi and hypertrophy of crypts occurred at the main site of infection in the anterior duodenum. The severity of villus atrophy was related to the number of infective larvae administered and/or the worm burden. In the ileum, beyond the main site of infection, hypertrophy of villi was only found in sheep receiving the greatest number of infective larvae.

KEY WORDS: Trichostrongylus colubriformis, Trichostrongylus vitrinus, concurrent infection, pathology, distribution.

Trichostrongylus species are ubiquitous parasites of sheep and are the source of significant production loss as well as death of the host if the intensity of infection is sufficiently high (Levine, 1986). The two principal species of Trichostrongylus found in domestic sheep, T. colubriformis and T. vitrinus (Levine, 1986; Dunn, 1969; de Chaneet & Dunsmore, 1988), have been studied extensively under laboratory conditions. Monospecific infections have been used to determine the dynamics of infection, distribution of nematodes in the small intestine and the pathological consequences of infection, for example, in the studies by Barker (1974, 1975a, b, c) on T. colubriformis and those by Coop et al. (1979), Taylor & Pearson (1979a, b), Taylor & Kilpatrick (1980) and Jackson et al. (1983) on T. vitrinus.

In the field, however, mixed infections rather than monospecific infections are common, though seasonal factors may affect the relative abundance of each species (Waller et al., 1981). There has been little investigation of the dynamics of mixed infections or of the effect of such infections on the host. Beveridge et al. (1989) in a comparative study of the pathogenicities of T. colubriformis, T. rugatus and T. vitrinus, included a group of sheep which were infected experimentally with all three species. They observed a posterior displacement of T. rugatus, suggesting that interaction...
occurred between the species, but their observations have not been pursued.

An experiment was therefore undertaken to examine the influence of the intensity of infection on the establishment and the distribution of *T. colubriformis* and *T. vitrinus* populations in pure and simultaneous mixed infections and the pathological consequences on the intestinal mucosa.

**MATERIALS AND METHODS**

**LAMBS**

Fifty-three Merino lambs were reared worm-free from birth in mesh-floored enclosures. Lambs received a diet of chopped lucerne hay fed *ad libitum*. Five sheep remained free of infection and constituted the control group. Groups of five or six sheep were infected with different numbers (7,500, 15,000 or 30,000 larvae) of *T. colubriformis* or *T. vitrinus* either as pure or mixed infections (Table 1) at six to seven months of age. When mixtures of larvae were used, larvae of the two species were administered at the same time. Three groups of sheep received 7,500, 15,000 or 30,000 larvae of *T. colubriformis*; three groups of sheep received 7,500, 15,000 or 30,000 larvae of *T. vitrinus*, and three groups of sheep received either a mixture of 7,500 larvae of *T. colubriformis* and 7,500 larvae of *T. vitrinus*, and three groups of sheep received either a mixture of 7,500 larvae of *T. colubriformis* and 7,500 larvae of *T. vitrinus*, and three groups of sheep received either a mixture of 7,500 larvae of *T. colubriformis* and 7,500 larvae of *T. vitrinus*, and three groups of sheep received either a mixture of 7,500 larvae of *T. vitrinus* or 30,000 larvae of *T. colubriformis* and 30,000 larvae of *T. vitrinus*.

**NEMATODES**

Third-stage larvae of *Trichostrongylus* spp. used for infection were the McMaster strain of *T. colubriformis* and the isolate of *T. vitrinus* utilised by Beveridge et al. (1989). Sheep were infected by stomach tube.

**PLASMA CONSTITUENTS**

Sheep were bled at weekly intervals for two weeks prior to infection and during the period of infection from the jugular vein. Plasma was separated and stored at -20°C. Plasma albumin and inorganic phosphorus concentrations were determined using a Cobas Mira (Roche Diagnostica).

**AUTOPSIES**

Sheep were killed 21 days post infection (PI) with an intravenous overdose of sodium barbital administered via the jugular vein. Immediately after death (within 3-5 minutes), the small intestine was removed and clamped, using paired haemostats, at one metre intervals from the pylorus for the first five metres and thereafter at intervals of four metres. A solution of 4% formalin and 1.5% glutaraldehyde in phosphate buffer was injected into the clamped areas (3-4 cm long) at 2, 4, 5 and 9 metres to fix tissues for histological examination. The metre or four metre long sections of intestine were then removed, washed with water to remove worms (three times) and the contents preserved in 10% buffered formalin for counting. The abomasum was also examined for nematodes.

**WORM COUNTS**

Worm counts were performed on a 10% aliquot of the material collected from each section. The aliquot was sedimented in water several times then stained with parasitological iodine and examined under a stereo-microscope. One hundred males or if fewer than 100, all males present, from each segment of intestine were removed, cleared in lactophenol and identified to species. This figure was used to determine the numbers of each species of nematode present in each section of the intestine, assuming that the species ratio for male worms was the same for female worms. The percentage of the total worm burden occurring in each metre of the intestine was also determined.

**HISTOLOGICAL EXAMINATIONS**

Histological samples taken at 2, 4, 5 and 9 metres from the pylorus were opened longitudinally, washed gently, stapled flat on a piece of cardboard, and fixed in 50 ml of the buffered formalin/glutaraldehyde mixture (pH 7.3) for 48 h, followed by storage in 10% buffered formalin. Fixed tissues were trimmed longitudinally and after dehydration, were embedded in paraffin wax and were sectioned at a thickness of 5 μm. The histological sections were then stained with haematoxylin and eosin.

For each segment and each sheep, two different measurements were made. The mean villus length was determined from 30 villi and the mean crypt depth was determined as the length between the base of the villi and the *lamina muscularis mucosae* assessed from 40 measurements. All measurements were performed on an Analytical Measuring System VIDS III (Shirehill Industrial Estate, Saffron Walden, UK).

**STATISTICAL ANALYSES**

Worm counts were transformed (ln (x + 1)) prior to analysis by one-way analysis of variance. Significant differences were considered to exist at the 5% probability level. Differences in distributions of nematodes within the intestine were determined by comparing the number of worms and the percentage of the total
worm burden in each segment of the gut by one-way analysis of variance. Histological measurements were analysed by one-way analysis of variance without transformation. Correlations were calculated between the mean numbers of worms in the proximal segments and the relative reduction in the size of villi in the second metre of the small intestine. Biochemical data were analysed using one-way analysis of variance and by analysis of covariance.

RESULTS

WORM COUNTS

All nematodes found were adults; no larval stages were encountered. No nematodes were found in the abomasum.

* T. colubriformis

Worm numbers (Table I). The mean establishment in groups of sheep infected only with *T. colubriformis* was between 26 and 55%. There was a statistically significant inverse linear relationship between percentage establishment and size of the infecting dose. Differences in percentage establishment between each rate of infection (30,000 versus 15,000 versus 7,500) were statistically significant. When mixed infections (*T. colubriformis* plus *T. vitrinus*) were compared with monospecific infections, there was a significant reduction in the establishment and/or development of *T. colubriformis* in the mixed infections when doses of 30,000 and 15,000 larvae were used, but not when 7,500 larvae were used. There was also a decline in percentage establishment with increasing size of infecting dose, with significant differences occurring between 30,000 versus 15,000 larvae and 15,000 versus 7,500 larvae.

Worm distribution (Fig. 1). In monospecific infections, the distribution of nematodes was similar, irrespective of the size of the infecting dose. Maximum numbers of worms were found in the first and second metres of the small intestine. The distribution of *T. colubriformis* and *T. vitrinus* was similar in monospecific infections, with the highest numbers in the first 1-5 metres and the lowest in the last 1-5 metres. The distribution of worms in the mixed infections was similar to that in monospecific infections.

Table I. – Experimental design and worm counts from sheep experimentally infected with 30,000, 15,000 or 7,500 third-stage larvae (L3) of *T. colubriformis* (C), *T. vitrinus* (V) or both species (V/C) and uninfected controls (U).

<table>
<thead>
<tr>
<th>Group no.</th>
<th>No. of sheep</th>
<th>Code</th>
<th>Infection schedule (Nos of L3)</th>
<th>T. colubriformis Geometric means (Percent establishment ±SD) (Ranges)</th>
<th>T. vitrinus Geometric means (Percent establishment ±SD) (Ranges)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>U</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>V/C 30,000</td>
<td>30,000</td>
<td>4,150(16±7) (1,550-7,260)</td>
<td>21,780(73±11) (16,400-26,160)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>V/C 15,000</td>
<td>15,000</td>
<td>3,800(26±7) (2,140-5,080)</td>
<td>12,200(82±16) (7,870-16,440)</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>V/C 7,500</td>
<td>7,500</td>
<td>3,700(50±13) (2,750-5,030)</td>
<td>6,780(88±12) (5,170-7,450)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>C 30,000</td>
<td>30,000</td>
<td>7,650(20±6) (5,800-9,800)</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>V 30,000</td>
<td>–</td>
<td>5,750(39±4) (5,000-6,600)</td>
<td>21,450(73±15) (16,110-27,170)</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>C 15,000</td>
<td>15,000</td>
<td>–</td>
<td>14,875(98±11) (12,220-16,520)</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>V 15,000</td>
<td>–</td>
<td>4,080(55±15) (2,180-5,610)</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>C 7,500</td>
<td>7,500</td>
<td>–</td>
<td>6,600(85±16) (4,620-7,500)</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>V 7,500</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*a, b, c, d, e, f: different individual superscripts indicate significant differences at 5 % level in mean numbers of *T. colubriformis*.

d, e, f: different superscripts indicate significant differences at the 5 % level in mean numbers of *T. vitrinus*.

Fig. 1. – Distribution of *Trichostrongylus colubriformis* and *T. vitrinus* in the small intestines of sheep experimentally infected with 30,000 (upper), 15,000 (mid) and 7,500 (lower) larvae of a single species.
(42-45 %) occurred in the first metre of the intestine, an equal or slightly smaller number in the second metre and much smaller, rapidly decreasing numbers in each subsequent metre. Less than 5 % of the total worm population was found in metre 5 and beyond of the small intestine. The major difference in worm distribution in mixed infection when compared with the monospecific infections occurred in the first metre of the intestine of groups infected with 30,000 larvae. In this case, the proportion of the nematode population in this segment of the intestine was significantly reduced and proportions in metre 4 were significantly increased (Fig. 2). In sheep infected with 15,000 and 7,500 larvae, there was an increase in the proportion of nematodes occurring in metre 4 of the intestine, but no significant reduction in the proportion of nematodes in the first metre.

* T. vitrinus

Worm numbers (Table I). An average of between 73 and 98 % of T. vitrinus larvae established in groups of sheep used for monospecific infections. There was no significant decrease in the percentage of nematodes that established following infection with 30,000 versus 15,000 larvae, or 15,000 versus 7,500 larvae. No significant differences in worm numbers were observed between monospecific and mixed infections at the same rate of infection. There were no significant differences in the percentage of larvae that established as the infective dose rate increased in the mixed infections.

Worm distribution. In monospecific infections, the distribution of nematodes was similar to that found in infections with T. colubriformis, with 42-48 % of nematodes in the first metre, declining to 1-10 % of nematodes by metre four (Fig. 1). No statistically significant differences occurred in the distribution of worms between monospecific and mixed infections or between different intensities of infection (Fig. 2).

**Histological Changes**

There was sub-total to total villus atrophy and severe crypt hypertrophy and dilation in infected sheep. The integrity of the epithelium was disrupted and enterocytes were cuboidal with a reduced brush border. In groups 2 (V/C 30 000) and 6 (V 30 000), three metres from the pylorus the villi remained stunted with shouldering and the villus-crypt ratio was less than one. At the same location, the villi of sheep in groups 3 (V/C 15 000 each) and 8 (V 15 000) did not appear to be as stunted as in groups 2 and 6 but the villus-crypt ratio remained less than one. The villi of all groups appeared to be similar distally from the fourth metre of the small intestine. Subjectively, from the fourth metre of the small intestine, there remained a mild increase in the cellularity of the lamina propria extending along the entire small intestine of infected sheep while all other parameters appeared to be similar to the uninfected group.

In the proximal region of the small intestine (section 2), villus atrophy was found in all the infected groups. Differences from controls in villus height were statistically significant (Fig. 3) in all groups except that infected with 7,500 T. colubriformis (Group 9). The severity of the lesions, i.e. the degree of villus atrophy, was related to the intensity of infection as the severest villus atrophy was associated with the largest infective doses of larvae (Fig. 3). Significant differences between infected groups in villus height were not present 4, 5 or 9 metres from the pylorus.

Sheep infected with T. vitrinus exhibited more severe villus atrophy than those infected with T. colubriformis (Fig. 3). To compare the histological damage due to monospecific or mixed infections, it was necessary to examine groups receiving the same total number of larvae, i.e. groups 3, 5 and 6 (total of 30,000 infective larvae) and group 4, 7 and 8 (total of 15,000 infective larvae). The decrease in villus height in mixed infections was intermediate between that found in pure infections of T. vitrinus and T. colubriformis.
The changes in villus sizes in the proximal part of the small intestine were associated with increases in crypt depth. Significant increases in crypt depth were found two metres from the pylorus following the administration of large numbers of larvae, i.e. 60,000 and 30,000 total numbers of larvae in mixed infection; 30,000 and 15,000 larvae in sheep receiving *T. vitrinus*, 30,000 larvae in sheep receiving *T. colubriformis* and 7,500 larvae in sheep receiving *T. vitrinus*. As with the villus changes, the extent of change in crypt depth was dependent on the number of parasites present whether the infection was pure or mixed (Fig. 3). No significant changes were evident 4, 5 and 9 metres from the pylorus.

In the distal part of the small intestine, differences in intestinal parameters were found between control and infected animals only in those sheep receiving the highest numbers of larvae. No significant differences were evident for villi and crypts 4.5 and 9 metres from the pylorus except in sheep infected with 30,000 larvae. In this group villi were significantly longer (23 %), 5 metres from the pylorus compared with control or other infected groups.

A significant correlation ($r = 0.96, df = 7; P < 0.02$) was observed between the arithmetic mean worm number present in the proximal region (metre 2) of the small intestine and the severity of villus atrophy (Fig. 4). This relationship was evident for monospecific infections with both *T. colubriformis* and *T. vitrinus* and also occurred with the total number of worms in the mixed infection. The slopes of the regressions were not significantly different whether the infections were pure or mixed.

**PLASMA CONSTITUENTS**

**Albumin concentrations**

In none of the pure infections of either *T. colubriformis* or *T. vitrinus* were there any significant differences from control values. With mixed infections, three weeks after infection, sheep infected with 30,000 *T. colubriformis* and 30,000 *T. vitrinus* (Group 2) had significantly lower albumin concentrations than the control sheep ($p = 0.0002$) (Fig. 5).

**Inorganic phosphorus concentrations**

The only significant differences from control sheep occurred three weeks after infection when Group 6 (30,000 *T. vitrinus*) had significantly lower concentrations than the controls ($p = 0.016$).
DISCUSSION

Substantial differences occurred in the overall rate of establishment of the two nematode species in pure infections in this experiment, with establishment of the *T. vitrinus* population ranging from 73 to 98% compared with 26% to 55% for *T. colubriformis*. Using the same isolates of nematodes, Beveridge *et al.* (1989) reported establishments of 37% and 44% of *T. colubriformis* and *T. vitrinus* respectively, suggesting that the establishment of *T. vitrinus* may have been unusually high in the present experiment and may have biased the results in favour of *T. vitrinus*. Simultaneous mixed infections with *T. colubriformis* and *T. vitrinus* suggested that the presence of *T. vitrinus* in some way inhibited the establishment and or development of *T. colubriformis* when 30,000 larvae of each species were administered. A similar reduction in establishment was observed when 15,000 larvae of each species were administered, but not when 7,500 larvae were used, suggesting that the effects observed are related to intensity of infection. Infections of *T. vitrinus* conversely were unaffected by the presence of *T. colubriformis* at all three intensities of infection. For *T. colubriformis*, there was a negative linear relationship between number of infecting larvae and percent establishment, though not in the case of *T. vitrinus*. Wagland *et al.* (1996) found no such relationship in sheep infected with 10,000 to 80,000 larvae of *T. colubriformis*. However, similar reductions in establishment with increasing numbers of larvae have been reported with other nematodes of ruminants, such as *Chabertia ovina* (see Herd, 1971).

Similar results were obtained whether the percentage establishment in mixed infections was calculated on the basis of the total number of larvae administered or the number of larvae of a given species. It was concluded that, in this experiment, *T. colubriformis* appeared to be more susceptible to the effects of increased intensity of infection than did *T. vitrinus*. The decline in its rate of establishment in mixed infections could be attributed either to density dependent effects or could be due to the presence of the congener. The data on establishment are explicable by the former hypothesis, apart from the apparent absence of any effect with infections of 7,500 larvae.

Both *T. colubriformis* and *T. vitrinus* occurred primarily in the duodenum in pure infections with 37-45% of nematodes occurring in each of the first two metres of the duodenum, confirming the results reported by Barker (1974) and Beveridge *et al.* (1989). The repeatability of these distribution patterns is illustrated in the groups of sheep in which pure infections were used. Because of this repeatability, deviations from it were considered to be biologically significant. Thus, the distribution of *T. colubriformis* was shifted posteriorly in mixed infections with 30,000 larvae of each species, but was apparently dependent on the intensity of infection, differences being detectable only in metre 4 when 15,000 larvae or 7,500 larvae of each species were administered.

These results differ from the earlier data of Beveridge *et al.* (1989) who infected sheep with 33,000 larvae each of *T. colubriformis*, *T. vitrinus* and *T. rugatus*. They reported no differences in the distributions of *T. colubriformis* and *T. vitrinus* compared with the controls, but observed a posterior displacement of *T. rugatus* instead. The intensities of infection were similar in the two experiments, sheep of the same breed and of similar age were used as well as the same strains of nematodes. The pattern of interaction between the two species may differ in some way from that observed when three species are present or the posterior displacement of *T. colubriformis* may be a less overt phenomenon and may not have been detected in the earlier experiment.

In spite of the increased intensity of infection, the distribution of nematodes within the intestine remained unaltered. This observation is of importance in comparing the simultaneous infection with 15,000 larvae of both *T. colubriformis* and *T. vitrinus* with that of single infections with 30,000 larvae of either species as the changed distribution in the simultaneous infections cannot be ascribed to a lack of vacant niches. Infections with greater than 60,000 larvae using the strains of nematodes employed in this experiment resulted in the deaths of sheep (unpublished observations) such that this aspect of the study could therefore not be explored further.
The lesions observed in the duodenum and upper jejunum of infected sheep in pure infections with *Trichos­­rongylus vitrinus* or *T. colubriformis*, that is subtotal villus atrophy and crypt hyperplasia, were less severe than the total atrophy described by Barker (1975a, b) in clinical trichostrongylosis, but were comparable to previous descriptions in sheep infected at a subclinical level with either *T. colubriformis* or *T. vitrinus* (Beveridge et al., 1989; Taylor & Pearson, 1979a, b; Roy et al., 1996). There was a strong correlation between the number of worms and the severity of villus atrophy at the site of parasitism. This was suggested by Barker (1974) based on semi-quantitative data in *T. colubriformis* infections. Our results showed that the relationship also occurred in *T. vitrinus* and in mixed infections. The similarity between correlation coefficients and gradients for both species suggests that the difference between the two species could be in fact directly related to differences in the establishment rates of the infection. The pathological consequences associated with mixed infections were intermediate compared with those of monospecific infections of the two different species and are probably related primarily to intensity of infection. Beveridge et al. (1989) concluded that *T. vitrinus* was more pathogenic than *T. colubriformis* based on a single experiment carried out with short term infections. They observed similar degrees of villus atrophy but greater disruption of the epithelium by *T. vitrinus* together with a greater reduction in plasma albumin concentrations and food intake. In the current experiment, no differences were observed in plasma albumin concentrations between groups of sheep infected with the two species, though the doses of infective larvae used (7,500-30,000) were lower than the 100,000 larvae used by Beveridge et al. (1989). Reductions of plasma albumin concentrations in the present experiment occurred primarily in mixed infections with the two species of nematodes in which a total of 60,000 larvae were used. The pathogenic effects appear to be related to the total numbers of nematodes which establish at a given site. The changes may be due to a direct (mechanical) effect of the nematodes or due to chemical secretions from the worms.

The presence of a distal adaptive region, characterised by an increased size of villi and crypts occurring beyond the main site of parasitism, has been described in *T. colubriformis* infection in sheep (Roy et al., 1996). In the present experiment, increases in villus height were found in sheep with mixed infections (60,000 larvae). In the sheep receiving monospecific *T. colubriformis* or *T. vitrinus* infections, some signs of villus hypertrophy were detected in the ileum but the extent of the phenomenon was not as marked as in the mixed infections. In mixed, as in monospecific infections, however, the distal changes were only detected at the highest levels of infection and no hypertrophy of mucosal structures was observed in the ileum of animals with low intensities of infection. These results suggest that the development of the adaptive, distal response could be dependent of the intensity of infection. Previous observations of experimental *T. colubriformis* infections in rabbits have also suggested that the distal response is density dependent (Hoste, 1989; Hoste et al., 1988; Hoste & Mallet, 1990).

Although the data presented suggest that interactions occur between *T. colubriformis* and *T. vitrinus* in the small intestine of sheep at high intensities of infection, the strains of both parasites used have been maintained in the laboratory for many generations. Before it can be concluded that the same phenomenon occurs in the field, additional experiments with newly isolated strains would be necessary. In addition, comparison between this and earlier experiments using the same strains of nematodes should be treated with caution due to possible changes in the laboratory strain of nematode as it undergoes sequential passages under highly artificial conditions.

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