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Response of Corriedale and Crioula Lanada Sheep to Artificial Primary Infection with *Haemonchus contortus*

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ABSTRACT

Clinical, parasitological and biochemical parameters were evaluated in Corriedale and Crioula Lanada sheep after a single experimental infection with *Haemonchus contortus*. Ten 4-month-old worm-free lambs, of each breed, were infected with 200 L_3 *H. contortus* per kg live weight and four uninfected animals of each breed were used as controls. Every week, the animals were weighed and blood and faecal samples were collected for measurement of packed cell volume (PCV), total serum protein (TSP) and albumin (ALB), and the number of eggs per gram of faeces (EPG), respectively. Twelve weeks after infection, the animals were slaughtered. The worm burden was determined and samples of the abomasal mucosa were processed for determination of the number of eosinophils, mast cells and globule leukocytes. No significant differences in PCV, TSP, ALB, parasite burden or the cell populations of the abomasal mucosa were observed between breeds, but Crioula lambs had a lower EPG count. The comparison of the infected groups with their respective controls revealed significant alterations in PCV, TSP and ALB in the Corriedale lambs and in PCV, TSP, ALB and the density of eosinophils and mast cells in the Crioula lambs.

Keywords: breed, burden, Corriedale, Crioula Lanada, *Haemonchus contortus*, helminthology, pathology, sheep

Abbreviations: ALB, albumin; EPG, number of eggs per gram of faeces; L_3, third-stage larvae; PCV, packed cell volume; TSP, total serum protein

INTRODUCTION

In the tropical and subtropical regions of Latin America, gastrointestinal nematodes of sheep now present elevated levels of anthelmintic resistance (Echevarria and Pinheiro, 1989; Echevarria *et al*., 1996; Eddi *et al*., 1996; Waller, 1997). It is currently known that anthelmintic resistance has been spreading geographically, both in terms of the number of resistant species and in terms of the variety of the anthelmintics involved (Sangster, 1999), indicating the necessity for studies on alternative or complementary methods for the control of these parasites, especially in small ruminants.

The observation that some breeds of sheep, and even some individuals of the same breed, are more resistant to *Haemonchus contortus* than the norm indicates that resistance to the parasite is genetically determined (Gray *et al.*, 1992). The selection of genetically resistant animals has, therefore, been adopted as one of the strategies for parasite control in countries such as Australia (Barger, 1993) and New Zealand (Niezen *et al.*, 1996).

Genetic resistance in sheep to gastrointestinal nematodes is mediated by an immune response and is based on the recognition of parasite antigens by the major histocompatibility complex (MHC) and stimulation of $CD4^+$ T lymphocytes. This stimulation leads to a cascade of events culminating in the genesis of mastocytosis and tissue and blood eosinophilia, and in the production of specific antibodies (Gill *et al.*, 1993), which, in theory, impair the establishment, development and fecundity of the helminths.

Thus far, no genetic markers for the resistance of sheep to gastrointestinal nematodes, in particular *H. contortus*, are available, so resistant animals have been selected on the basis of phenotypic markers. According to Douch and colleagues (1996), resistant animals can be defined using parasitological, immunological and/or production parameters. The most widely employed parameters for ante-mortem evaluation are the number of eggs per gram of faeces (EPG), blood eosinophilia, concentration of antibodies, cytokines and mediators released by mast cells, and production parameters, such as weight gain and meat and wool production. The parasite burden and the granulocytic response in the tissues are parameters used for post-mortem evaluation.

Breeds of sheep considered to be resistant on the basis of these parameters are the Florida Native (Courtney *et al.*, 1985; Amarante *et al.*, 1999), St. Croix (Zajac *et al.*, 1990; Gamble and Zajac, 1992), Red Maasai (Mugambi *et al.*, 1996; Wanyangu *et al.*, 1997) and Gulf Coast Native (Bahirathan *et al.*, 1996; Miller *et al.*, 1998).

Crioula Lanada sheep, reared in Southern Brazil, probably originated from animals of different breeds, including the Churra breed, which were brought to South America by colonizers soon after discovery. These animals were gradually replaced by more specialized breeds since, in addition to undesired characteristics such as the presence of horns and long and thick fur, they presented a low performance in meat and wool production (Vaz, 1993). For several centuries, these animals procreated freely in a semi-wild fashion as they became adapted to the local environment by exposure to the climatic and nutritional contingencies (Jardim, 1987). Some preliminary studies have shown that these animals are highly resistant to gastrointestinal helminths (Borba *et al.*, 1997).

Based on the above considerations, the objective of the present study was to compare the haematological, biochemical, parasitological and histological alterations in Crioula Lanada and Corriedale sheep after artificial primary infection with *H. contortus*.

MATERIAL AND METHODS

Animals

Twenty-eight lambs, 14 Corriedale and 14 of the local Crioula Lanada breed, from Embrapa, Pecuária Sul, Bagé, Brazil, born in September 1997 were used. The Corriedale lambs were descendants of two sires and the Crioula Lanada of seven. At 2 days of age, the lambs were separated from their mothers and housed. They were at first fed on cow's milk, which was gradually replaced by a solid diet. At 3 months of age, the animals were weaned and maintained on a diet consisting of 90% alfalfa hay (*Medicago sativa*) and 10% of a commercial ration (Cobagelã, Bagé, Brazil) containing about 17.0% crude protein and 65.0% total digestive nutrients (TDN).

Experimental design

At approximately 3 months of age, the animals were randomly allocated to four groups. Ten lambs of each breed were allocated to the infected groups COR-I (7 males and 3 females) and CRI-I (6 males and 4 females) and were each orally inoculated with 200 L_3 of *H. contortus* per kg live body weight in a single dose. Four lambs (2 males and 2 females) of each breed were not infected and served as the control groups COR-C and CRI-C.

The animals were weighed at the time of infection to determine the larval dose, and then every 14 days until the end of the experiment for the determination of weight gain. Blood samples, taken from the jugular vein, and faeces from the rectal ampulla were collected weekly for the determination of packed cell volume (PCV) and EPG counts, respectively.

Parasites

Infective *H. contortus* larvae (L_3), sensitive to anthelmintics (Echevarria *et al.*, 1991), were obtained from the Laboratório de Helmintologia da Embrapa, Pecuária Sul, Brazil, and used to infect the experimental animals. The infective larvae were obtained from donor animal faeces, cultured in an incubator at 27°C for 7 days according to the method of Roberts and O'Sullivan (1950), and stored at 4°C until the time of use.

Parasitology and necropsy

The EPG count was determined by a method modified from that of Gordon and Whitlock (1939) and faecal cultures for generic identification of the parasites were carried out as described by Roberts and O'Sullivan (1950).

Twelve weeks after infection, the lambs were slaughtered and necropsied. The abomasum was opened along the greater curvature and washed out for parasite collection. A 10% aliquot was fixed with 5% formalin for later identification of the

parasites. Before washing out the abomasum, 2 cm^2 mucosal fragments were obtained and fixed in 10% neutral buffered formalin for histological examination. The fragments were processed by routine histological methods and embedded in Histosec (Merck, Darmstadt, Germany). Sections of 5–7 μm were cut with a steel knife microtome and stained with haematoxylin–eosin or toluidine blue.

Haematology

The PCV was measured using the microhaematocrit method. Total serum protein and albumin concentrations were determined at two-week intervals by the modified colorimetric method of Goodwin using the Total Protein and Albumin LABTEST kit (System for Clinical Diagnosis, Labtest Diagnóstica SA, Lagoa Santa, Brazil).

Histology

The numbers of eosinophils and mast cells were determined in sections stained with haematoxylin–eosin and toluidine blue, respectively; the cells in 15 randomly chosen fields from the muscularis to the mucosal surface being counted (Huntley *et al*., 1992). The number of globule leukocytes was determined in sections stained with haematoxylin–eosin, using a Zeiss microscope equipped with a mercury vapour lamp (HBO 200), the cells from 15 fields being counted as described above. The results of all cell counts are reported as the mean number of cells per mm^2 of mucosa.

Statistical analysis

The breeds were compared in terms of the EPG, PCV, total protein, albumin, live weight and the number of cells in the mucosa by analysis of variance (GLM procedure) using the SAS Statistical Package (SAS, 1989). Means were fitted using the least-squares method. The effects included breed, period (0–12 weeks), treatment (infected and control) and the breed × period, breed × treatment, period × treatment, and breed × period × treatment interactions. Nonsignificant interactions were excluded from the model. The EPG values were $\log_{10}(n+1)$-transformed to stabilize the variance. Comparison of the worm burdens between the breeds was performed by the Mann–Whitney test using the Minitab Version 11 software (Minitab INC, State College, PA, USA). Values are presented as mean ± SD.

RESULTS

Corriedale lambs had higher weights than Crioula lambs from the beginning to the end of the experiment ($p < 0.01$). The final weight gain, however, was similar for both breeds ($p > 0.05$) (Figure 1).

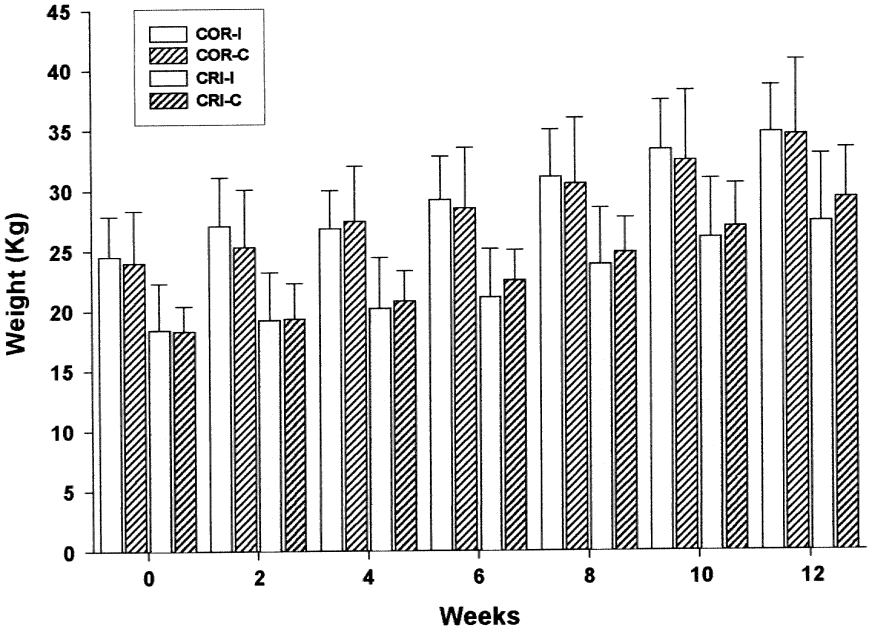

Figure 1. Mean and standard deviation values of the live weight of Corriedale and Crioula Lanada lambs infected with *Haemonchus contortus* (COR-I and CRI-I) and uninfected controls (COR-C and CRI-C)

The initial PCV values obtained for all sheep were within the range 30–45% and the observed differences were not significant (Figure 2). The values gradually decreased in the infected lambs of both breeds, reaching minima of 25% and 27% during the fourth week (COR-I) and third week (CRI-I), respectively. Although the CRI-I animals recovered mean PCV values of 30% during the sixth week, while the COR-I animals did so only during the twelfth week of the experiment, the differences between the breeds were not significant ($p > 0.05$). However, the PCV values were significantly lower in the infected animals of both breeds compared to their respective controls ($p < 0.01$).

The initial total serum protein concentration was 6.0–7.5 g/dl and that of albumin was 2.4–3.0 g/dl, with no significant difference between the breeds. After infection, the total protein decreased gradually to reach a minimum of 4.98 ± 0.34 g/dl in the Crioula lambs and 5.14 ± 0.25 g/dl in the Corriedale lambs, with again no significant difference between the breeds. Albumin was reduced similarly in both infected groups, reaching minimal values of 2.70 ± 0.28 g/dl and 2.74 ± 0.18 g/dl in the Crioula and Corriedale lambs, respectively. Both the total protein and albumin were significantly lower in infected animals than in their respective controls ($p < 0.01$).

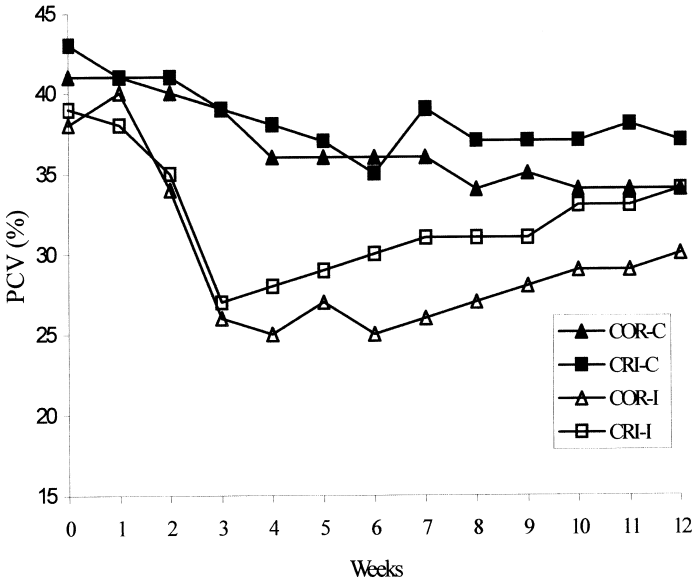

Figure 2. Mean packed cell volume (PCV) of Corriedale and Crioula Lanada lambs infected with *Haemonchus contortus* (COR-I and CRI-I) and uninfected controls (COR-C and CRI-C)

The first eggs were detected in faeces on day 21 after infection. The peaks of egg elimination occurred during the fourth and fifth week for the Crioula and Corriedale sheep, respectively, declining thereafter until the end of the experiment (Figure 3). Values of EPG were significantly lower for the Crioula sheep than for the Corriedale animals ($p < 0.01$). The control animals tested negative for nematode eggs throughout the experimental period.

The number of *H. contortus* specimens recovered at autopsy ranged from 0 to 3010 for the Corriedale sheep and from 0 to 1720 for the Crioula sheep (Table I). The median value of the worm counts obtained for the Corriedale sheep (1145) was higher than that for the Crioula animals (280), but this difference was not significant ($p > 0.05$).

The mean number and standard deviations of the eosinophils, mast cells and globule leukocytes in the abomasal mucosa, expressed as the number of cells per mm^2, are shown in Table II. The numbers of eosinophils, mast cells and globule leukocytes were generally higher in the infected animals than in their respective controls. However, these differences were only significant for the number of eosinophils and mast cells in the Crioula animals ($p < 0.05$).

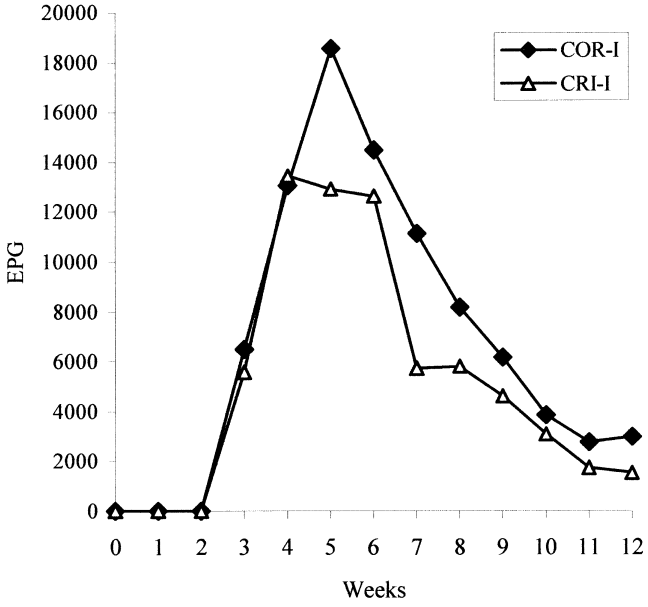

Figure 3. Mean faecal egg counts (EPG) for Corriedale (COR-I) and Crioula Lanada (CRI-I) lambs infected with *Haemonchus contortus*

TABLE I
Numbers of male and female *H. contortus* from Corriedale and Crioula lambs experimentally infected with 200 infective larvae/kg live weight

	Corriedale			Crioula		
Lamb	Males	Females	Total	Males	Females	Total
1	720	1150	1870	–	–	–
2	780	780	1560	850	870	1720
3	100	380	480	800	720	1520
4	380	410	790	90	160	250
5	1850	1160	3010	340	80	420
6	0	0	0	260	190	450
7	420	500	920	0	0	0
8	1060	1880	2940	30	60	90
9	0	0	0	70	60	130
10	530	840	1370	180	100	280
Mean	584	710	1294	291	249	540

TABLE II
Mean($\pm$SD) numbers of eosinophils, mast cells, and globule leukocytes per mm^2 of abomasal mucosa of Corriedale and Crioula Lanada lambs infected with *H. contortus* (COR-I and CRI-I) and uninfected controls (COR-C and CRI-C)

Group	Eosinophils	Mast cells	Globule leukocytes
COR-C	$1.39^{a} \pm 6.78$	$12.78^{a} \pm 10.55$	$47.06^{a} \pm 60.95$
COR-I	$10.22^{a} \pm 4.29$	$37.17^{a} \pm 6.67$	$177.64^{a} \pm 38.55$
CRI-C	$0.55^{a} \pm 6.78$	$13.33^{a} \pm 10.55$	$126.47^{a} \pm 60.95$
CRI-I	$17.79^{b} \pm 4.29$	$40.60^{b} \pm 6.67$	$212.95^{a} \pm 38.55$

[a,b]In each column, results with different superscripts are significantly different ($p < 0.05$)

DISCUSSION

The typical clinical signs of *H. contortus* infection in sheep, such as anorexia, apathy, prostration and submandibular oedema were not observed in the infected animals of either breed, thus characterizing the infection as subclinical. Furthermore, no interference of the infection with the weight gain of the animals was observed throughout the experiment, suggesting that the animals' diet may have been, at least in part, responsible for the absence of signs. Several studies have shown that sheep fed an additional amount of protein or urea can better withstand the pathogenic effects of parasitism. Abbott and colleagues (1986) observed that lambs infected with 350 *H. contortus* larvae per kg weight and fed a diet containing 16.9% or 8.8% protein showed similar numbers of eggs per gram and parasite burden, but only those receiving the low-protein diet presented lack of appetite, weight loss and elevated mortality.

Another factor that might have contributed to the absence of clinical manifestations was the larval dose of 200 L_3 per kg live weight used for infection. Abbott and colleagues (1985, 1986), using two different doses of *H. contortus* to infect sheep, observed that a dose of 125 L_3/kg live weight produced only a moderate infection, with parasite burdens ranging from 0 to 900 parasites and few alterations in haematological or biochemical values.

The comparison of haematological and biochemical parameters (PCV, total serum protein and albumin) between breeds revealed only very slight differences, which were only significant when the infected lambs were compared to their respective controls. The most significant changes coincided with the period during which the highest EPG values were observed.

According to Douch and colleagues (1996), the EPG is the parameter showing the best correlation with parasite burden in sheep, especially in young animals, and most experimental selections for resistance are based on this parameter. However, the results obtained by various authors studying primary infections with *H. contortus* reveal that

the behaviour of different breeds in terms of the number of eggs per gram in the faeces is variable. Some studies have shown significant differences in these numbers (Radhakrishnan *et al.*, 1972; Bradley *et al.*, 1973; Zajac *et al.*, 1990), as was also observed in the present investigation, while in other studies the differences were not significant (Courtney *et al.*, 1985; Gamble and Zajac, 1992). Courtney and colleagues (1985) even pointed out that the differences in the EPG are mainly due to the acquired immune response and that the differences between breeds are small and highly variable during primary infections.

The median value of the worm counts in the Crioula group was approximately four times lower than the median number in the Corriedale lambs, indicating higher resistance in the former breed. The intrabreed variability, which may be high during primary infections (Zajac *et al.*, 1990; Gill, 1991), and the small number of animals used in the present study might be the cause for the difference being only close to significance.

The frequently observed association between elevated granulocyte numbers and nematode resistance has led to more detailed investigations on the possibility of using this parameter as a phenotypic marker for resistance. Some studies have shown that the increase in eosinophils in the mucosa is more marked in animals selected for low EPG values than in those with high EPG values (Rothwell *et al.*, 1993; Stevenson *et al.*, 1994). Similarly, an increase in mast cells and globule leukocytes is considered to be a prominent factor in resistant animals. In the present study, the number of eosinophils, mast cells and globule leukocytes was similar in the animals of both breeds ($p > 0.05$). However, it should be noted that, for the Crioula sheep, the number of mast cells and eosinophils was significantly higher in infected animals, whereas this was not the case in the Corriedales, possibly because of the light infections used.

According to Huntley and colleagues (1992), the differentiation and proliferation of mast cells and globule leukocytes, which are related, require continuous and repeated antigenic stimulation. We suspect that, in the present study, the stimulus was not sufficient to cause marked proliferation of these cells since the animals were exposed to a single, relatively light, experimental infection. Gill (1991) and Zajac and colleagues (1990), studying experimental infections with *H. contortus*, obtained similar results with sheep exposed to only a primary infection. Amarante and colleagues (1999) also observed no differences in the eosinophil, mast cell or globule leukocyte populations in Florida Native and Rambouillet sheep, or in their F_1 and F_2 generations, exposed to two natural infections and killed 30 days after the last artificial challenge, suggesting that there had already been a reduction in the number of cells in resistant sheep by that time.

Although the present results are not sufficient to confirm that Crioula sheep are more resistant to *H. contortus* infection than Corriedales, some of the data point in this direction and deserve further investigation. Among the phenotypic parameters analysed, the number of eggs per gram, considered to be one of the most indicative markers for resistance, was significantly lower in the animals of this breed. In addition, although not statistically significant, other data such as the tissue response and parasite burden were relatively favourable in the Crioula sheep, considering that these animals had been infected at an age at which they are not considered to be strongly immunocompetent (Douch *et al.*, 1984).

Genetic markers for resistance to helminths may be available in the near future and it will then be important to obtain native flocks whose genomes possess these genes at high frequency for crossings. Further studies are necessary to determine whether Crioula sheep represent such a reserve of genes for resistance to trichostrongylid parasites.

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