



A Th2 type of immune response is associated with increased resistance to *Haemonchus contortus* in naturally infected Gulf Coast Native lambs

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ABSTRACT

Haemonchus contortus is one of the major nematode parasites causing substantial economic losses in small ruminant farming worldwide. Recently, effectiveness of anthelmintic treatment has decreased due to an increasing problem of nematode populations that have developed resistance to anthelmintics. Efforts to develop effective vaccines have had limited success. There are certain breeds of sheep that are relatively resistant to the parasite including Gulf Coast Native (Native) sheep. Understanding the protective nature of the immune response that helps these breeds of sheep control infection could enable the development of vaccines to enhance control programs. This experiment was designed to compare the immunological responses of resistant Native versus susceptible Suffolk sheep in order to identify the mechanisms responsible for this resistance. Immune responses were evaluated in naturally infected Native and Suffolk lambs that grazed pasture contaminated predominantly with *H. contortus*. Ten lambs of each breed grazed together for 42 days. Fecal, blood and serum samples were collected on 0, 2, 4, 7, 10, 14, 21, 28, 35 and 42 days of exposure. Five lambs of each breed were necropsied on day 35 and five on day 42 for nematode recovery and abomasal tissue sample collection. Throughout the course of infection, Native lambs had significantly lower FEC, significantly lower PCV reduction percent, and significantly higher serum IgE after day 14 and increased expression of IL-4 on day 10 post-exposure compared to Suffolk lambs. At both necropsy time points, Native lambs had significantly greater numbers of mucosal mast cells, eosinophils and globule leukocytes in abomasal mucosa than Suffolk lambs. Results indicated that Native lambs had a more pronounced immune response to infection with *H. contortus* than Suffolk lambs which may be responsible for the observed resistance to infection.

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1. Introduction

Gulf Coast Native (Native) sheep are known to thrive well in the warm and humid climate of Southeastern US where *Haemonchus contortus* is a common nematode parasite affecting small ruminants. These sheep have

demonstrated resistant characteristics against *H. contortus* compared with the Suffolk breed of sheep in suckling lambs and mature ewes (Bahirathan et al., 1996; Miller et al., 1998). Furthermore, Native lambs were found to possess an immunologic component to their resistance characteristics against *H. contortus* in that treatment with dexamethasone and antiCD4+ T cell antibody decreased resistance (Pena et al., 2004, 2006). Cross-breeding of Native lambs with Suffolk lambs also indicated that resistance is genetically controlled and highly variable

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among individuals within the breed (Miller et al., 2006). However, the components that are responsible for this resistance were not characterized.

Repeated infections of lambs with *H. contortus* induce partial resistance resulting in increased prepatent period, lowered fecal egg count (FEC) and fewer worm recoveries (Gomez-Munoz et al., 1999; Lacroux et al., 2006). Resistant lambs demonstrate induction of Th2 type of cytokines especially IL-4, IL-5 and IL-13 mRNA expression in abomasal and mesenteric lymph node cells (Terefe et al., 2007), with repeatedly infected lambs expressing cytokine mRNA earlier during an infection than the primary infected lambs. Other cytokines such as IFN- γ , IL-10, IL-12, TNF- α and eotaxins did not seem to have been induced. Cytokine levels produced by abomasal lymph node (ALN) and mesenteric lymph node (MLN) cells, measured by Gill et al. (2000), showed that IFN- γ was substantially reduced compared to uninfected controls on days 5 and 28 post-infection while IL-5 was significantly increased in infected cell cultures. However, the ALN lymphocytes stimulated with *H. contortus* intestinal antigens expressed IL-13 compared with the principle induction of IL-4 and IFN- γ by concanavalin A (ConA) (Jasmer et al., 2007).

Mucosal mast cells, eosinophils, globule leucocytes, and various immunoglobulins are commonly measured components of parasite immunity. While some studies have found cellular components to play a significant role in resistance (Lacroux et al., 2006; Terefe et al., 2007), others have not (Amarante et al., 1999); however, the latter found a negative correlation with worm burden and FEC. Similar findings were also published regarding the roles of immunoglobulins. Gomez-Munoz et al. (1999) have reported no correlation between the serum levels of IgG, IgG1, IgG2, IgM and IgA and resistance. However, increased serum levels of IgG1 and IgG2 with decreased FEC were reported by Schallig et al. (1995) and IgG1 and IgE by Gill et al. (2000) in resistant sheep. In another study, Schallig et al. (1994) reported IgA specific to L3 and IgG1 and IgG2 response to *H. contortus* adult antigens.

In the case of other trichostrongyle infections (such as *Trichostrongylus axei*), mucosal mastocytosis, globule leukocytosis, and eosinophilia were the prominent features accompanied by increased inhibition of larval migration in abomasal mucosa of repeatedly exposed sheep compared to the controls (Pfeffer et al., 1996). Likewise, increased serum levels of *T. axei*-specific IgG and IgG1 with a peripheral eosinophilia were reported in these sheep. Similarly, sheep immunized by infection with *Teladorsagia circumcincta*, and *Trichostrongylus colubriformis* demonstrated increased peripheral and tissue eosinophil numbers and increased tissue globule leukocytes in the abomasum (Stankiewicz et al., 1995).

This study was designed to evaluate various components of the immune response to *H. contortus* in pasture-infected relatively resistant Native versus susceptible Suffolk lambs. The results indicate that a Th2 type of immune response is associated with increased resistance in Native lambs.

2. Materials and methods

2.1. Location and animals

The experiment was conducted at the Small Ruminant Farm, Louisiana State University Agricultural Experiment Station, Central Research Station, Baton Rouge, LA 70803. Ten 5–6-month-old Native and Suffolk lambs were randomly selected. The experimental protocol was approved by the Louisiana State University Institutional Animal Care and Use Committee.

2.2. Experimental design and sampling scheme

The lambs were repeatedly treated with levamisole (AgriL[®], St. Joseph, MO) and albendazole (Valbazen[®], Pfizer, New York, NY) at 12 mg/kg and 20 mg/kg body weight per orum respectively, for two consecutive days each week for 3 weeks until fecal egg counts (FEC) were zero or close to zero. The lambs were housed on a dirt floor shed at night and were allowed to graze in a multi-parasitic nematode species-contaminated pasture; where *H. contortus* was the most prevalent parasite species. Fecal samples were collected weekly for FEC. Peripheral blood samples were collected for the determination of packed cell volume (PCV), differential leukocyte count, RNA extraction and serum on days 0, 2, 4, 7, 10, 14 and weekly then after. Gut wash samples, abomasal tissue samples and lymph nodes were collected at the time of necropsy on days 35 and 42 post-infection.

2.3. Parasitological parameters

2.3.1. FEC and parasite recovery

Weekly FEC was determined by a modified McMaster technique as describe by Whitlock (1948). The samples with a FEC of 0 egg per gram (epg) were subjected to a sugar floatation technique.

Parasites were recovered from 100 ml of wash and soak samples from abomasa and small intestines after straining through a 200 μ m pore size strainer. The first 100 worms were handpicked and preserved in lactophenol for subsequent speciation and the rest were counted and recorded. When there were less than 10 worms in the first aliquot, a second aliquot of 100 ml was examined. All the 500 ml of samples from large intestines was examined. The number obtained was extrapolated to estimate the total number of worms in each organ.

2.3.2. Whole worm antigen (WWA) preparation

WWA was prepared using adult *H. contortus* worms collected from lambs used in this experiment and preserved at -20°C . Worms were handpicked and washed in phosphate buffered saline (PBS) solution several times. The whole worms were ground and homogenized in 25 ml of PBS using a Tissumizer (Tekmar Co., Cincinnati, OH). The homogenate was centrifuged at $5000 \times g$ for 20 min and the supernatant was collected (Kabagambe, 1997). The soluble protein concentration in the supernatant solution was determined by a microtiter plate-adapted bicincho-ninic acid assay (Pierce, Rockford, IL).

2.4. Systemic parameters

2.4.1. Blood packed cell volume (PCV) and differential leukocyte count

Fresh blood was collected on days 0, 2, 4, 7, 10, 14, 21, 28, 35 and 42 post-exposure by jugular venipuncture into EDTA-coated vacutainer tubes (Becton, Dickinson, & Co.). A thin blood smear was prepared on clean dry microscopic glass slides. Dried and fixed blood smears were stained by Wright–Giemsa method and leukocytes were enumerated under oil immersion microscopy. PCV was determined by a microhematocrit method using an Autocrit Ultra 3 Microhematocrit Centrifuge (Becton, Dickinson, & Co.).

2.4.2. RNA extraction and quantitative polymerase chain reaction (qPCR) analysis for cytokine mRNA expression

Two and a half millilitre of blood was drawn by jugular venipuncture into a 3 ml plastic syringe and transferred to Paxgene blood RNA tubes (Qiagen) containing cytolitic and RNA stabilizing reagents. The content was mixed thoroughly by inverting at least 10 times and were stored at -20°C until processed further.

Total RNA in each peripheral blood sample was extracted using a RNA extraction kit (Paxgene and Versagene), according to the manufacturer's recommendation. The total amount of RNA extracted from each sample was determined by spectrophotometric reading of absorbency and the 260/280 ratios were greater than 1.8 for all samples. Complementary DNA (cDNA) strand was synthesized with $1\text{ }\mu\text{g}$ of RNA reverse transcribed in an $80\text{ }\mu\text{l}$ reaction containing 20 unit AMV RTase, $0.5\text{ }\mu\text{g}$ oligo dT primers, 40 unit of rnasin and 5 mM MgCl_2 (Promega, Madison, WI) (Horohov et al., 2005). The temperature and cycle length for cDNA synthesis were 42°C for 15 min, followed by 95°C for 5 min, and 4°C for 15 min.

Forward and reverse primer sequences for cytokines IL-4, IFN- γ and IL-10 mRNA (Table 1) were purchased from IDT (Coralville, IA), based on previously published sequences for ovine IL-4, IL-10 and IFN- γ (Hein et al., 2004) and the housekeeping gene ATPase (Budhia et al., 2006).

Cytokine mRNA expression was detected by real-time PCR using the My-iQ single color real-time PCR detection system (BioRad). The reactions were carried out in $25\text{ }\mu\text{l}$ volumes in duplicate in 96 well PCR plates as described by Budhia et al. (2006). Briefly, iQTMSYBR[®] Green Supermix (BioRad) containing dNTP, iTaq DNA polymerase, SYBR Green I, fluorescein and KCl, Tris-HCl and MgCl_2 , $1\text{ }\mu\text{M}$ each of reverse and forward primers, and $5\text{ }\mu\text{l}$ of cDNA template were added to each plate. Pooled non-reverse transcribed template and blank samples were used in duplicate in each plate as negative controls. The amplifica-

tion protocol included preheating to 95°C for 10 min followed by 40 cycles of 95°C for 20 s, for annealing, and 45 s for extension at 60°C . It also included a final incubation of 1 min each of annealing and extension at 95°C and 55°C , respectively. Melting curves were generated at the end of the each run by running the product through temperature 55°C and increasing by 0.5°C after every 15 s until the final temperature of 95°C was reached.

Cycle thresholds (Ct) were calculated by the Mycycler software (mean ± 10 times the standard deviation of background) to determine the threshold values. An arbitrary cut-off point was made if values were within two cycles of the corresponding RT negative samples on a plate. Each gene was analyzed individually and the highest threshold required to accurately analyzing all the genes in that plate was used. Automatic baseline settings were used throughout the analysis.

Relative expression was determined using the $\delta\delta\text{Ct}$ method (Livak and Schmittgen, 2001). The mean Ct for each sample was calculated for the duplicate wells, and δCt s were obtained by subtracting the Ct for housekeeping gene ATPase, from the Ct's for each cytokine gene being analyzed. Outlier samples were ignored when traces showed clear irregularities. Some of the samples had ATPase detection delayed indicating a poor cDNA yield causing the genes of interest to be delayed in their detection. In order to avoid over-estimation of the amount of cytokine in target, the samples with low levels of ATPase expression were discarded from analysis. $\delta\delta\text{Ct}$ s were calculated using a calibrator consisting of the mean of all the individual δCt s at the initial sampling time point and subtracting it from the individual δCt s. The relative quantity (RQ) value for each mRNA level was calculated as $2^{-\delta\delta\text{Ct}}$.

2.4.3. Immunoglobulin isotype profile

Peripheral blood samples were collected by jugular venipuncture in 10 ml vacutainer tubes (No Additive, Becton, Dickinson, & Co.) and serum was separated by centrifugation for 10 min at 1000 rpm. The serum was transferred into 5 ml Eppendorf tubes and preserved at -20°C until analyzed by ELISA.

High binding polystyrene plates (Costar EIA/RIA, Corning, Inc.) were coated with $100\text{ }\mu\text{l}$ of *H. contortus* WWA in 0.05 M carbonate bicarbonate buffer, pH 9.6 (Sigma–Aldrich Co.) with a concentration of $5\text{ }\mu\text{g/ml}$. The plates were sealed with a microtiter plate sealer (SealPlate Microplate Adhesive Film, E & K Scientific) and refrigerated overnight for coating. The plates were washed four times with phosphate buffered saline (PBS, Sigma–Aldrich Co.) solution and patted dry. The wells were blocked for 5 min

Table 1
Primer sequences for ovine cytokines and housekeeping gene used for SYBR Green real-time PCR.

Primer name	Forward sequence (5'–3')	Reverse sequence (5'–3')
OvATPase	TTCCTACTGCCCTGGATG	CACGAAGATGAGAAGCGAGT
OvIL-4	GGAGCTGCCTGTAGCAGACG	TTCTCAGITGCGTTCTTTGGG
OvIL-10	TGCTGGATGACITTAAGGGTTACC	TCAATTCCGACAAGGCTTGG
OvIFN- γ	TGA TTCAAATTCGGTGGATG	TTCAITGATGGCTTTGGCG

with 200 μ l of blocking buffer (Starting Block (PBS) Blocking Buffer, Pierce Biotechnology) then emptied. Serum samples were diluted 1:25 in Starting Block T20 (PBS) Blocking Buffer (Pierce Biotechnology). One hundred microlitres of diluted serum sample, appropriate positive and negative control sera were added in duplicate to each well in the plate, sealed and incubated at room temperature for 1 h. Plates were washed four times with PBS and patted dry. For total IgG, 100 μ l of 1:25,000 rabbit anti-goat IgG horse radish peroxidase (Sigma–Aldrich Co.) in Starting Block T20 blocking buffer was added per well and incubated for 1 h before adding chromagen (1-Step Ultra TMB-ELISA; Pierce Biotechnology) substrate. For isotypes IgG1, IgG2, IgM, IgA, and IgE (provided by CSIRO, Armidate, NSW, Australia); 100 μ l of 1:5 mouse anti-sheep IgG1, IgA and IgE and 1:10 IgG2 and IgM, monoclonal antibodies were added and incubated at room temperature for 1 h. Afterwards, 100 μ l of 1:25,000 goat anti-mouse IgG conjugated with horse radish peroxidase was added to each well after washing four times with PBS solution. Plates were washed four times with PBS solution and patted dry and 100 μ l/well of the chromagen substrate, 1-Step Ultra TMB-ELISA (Pierce Biotechnology) was added and incubated without a seal for 30 min in a dark cabinet at room temperature for isotype IgM while it was 1 h for all other isotypes. The reaction was stopped with the addition of 100 μ l/well of 2 M H_2SO_4 . Absorbencies were read at 450 nm by an ELISA plate reader (ELX800 Universal Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT) and are presented as optical density (OD) for each sample.

2.5. Histopathological examination

At necropsy, the abomasal and prescapular lymph nodes were removed as soon as the abdominal cavity was opened. Approximately 2 cm $\times$ 2 cm mucosal pieces were taken from the cardiac, fundic and pyloric regions of the abomasum and fixed in 10% buffered formalin solution. Fixed abomasal tissue samples were trimmed, embedded in paraffin and sectioned as 3 μ m thick. Eosinophils, neutrophils and globule leukocytes were enumerated in sections stained with hematoxylin and eosin (H&E) while mucosal mast cells were counted in sections stained with toluidine blue. The staining was performed using a Leica TP 1050 Automatic Tissue Processor and Leica Autostainer XL staining procedure. The reagents used were hematoxylin (Anatech, Ltd., Battle Creek, MI), eosin Y (Anatech, Ltd., Battle Creek, MI), Propar-Anatech, Ltd., Battle Creek, MI) and absolute alcohol (AAPER Alcohol and Chemical Co., Shelbyville, KY). The number of cells in each of ten 0.3 mm $\times$ 0.3 mm fields were counted in the lamina propria of the three respective regions of the abomasum and recorded as the mean number per mm².

2.6. Statistical analysis

The data obtained were analyzed by the SAS statistical package version 9.1.3. FEC and parasite recovery data were log transformed to stabilize variance. Repeated measures analysis of variance was used for comparisons of FEC, PCV

reduction percentage, peripheral eosinophil percentage, immunoglobulin (ELISA OD) and cytokine mRNA expression levels over each sampling time point with split-plot arrangement of treatments with treatment group and animals within treatment groups as main plot effects and time by group interaction as subplot effects. When a significant difference overall was found, a Scheffé's test was used for main effect with pair wise comparison. Parasite recovery and abomasal cell enumeration data were compared by unpaired Student *t*-test. For all the tests, a *p*-value of ≤ 0.05 was set as the level of significance.

3. Results

3.1. Parasitological parameters

3.1.1. FEC

Mean FEC is presented in Fig. 1. The prior nematode infection was not completely cleared despite repeated administration of anthelmintics and the animals continued to show a low FEC. However, trichostrongyle type ova increased significantly in both the groups 3 weeks post-exposure and the increase in Suffolk lambs was significantly ($p < 0.05$) higher compared to Native lambs through day 42.

3.1.2. Parasite recovery

H. contortus was the only species recovered from the abomasum (Fig. 2). About sixfold greater numbers ($p < 0.05$) of *H. contortus* were recovered in Suffolk compared to Native lambs on day 35 and over fourfold greater ($p < 0.05$) on day 42 post-exposure. *Cooperia* and *Trichuris* spp. were the two other parasitic nematode species recovered from small and large intestines. The mean number of *Cooperia* spp. recovered from small intestines was 990 and 715 in Native and Suffolk lambs, respectively, on day 35. There were fewer recovered (250 and 240, respectively) on day 42 post-exposure. The mean number of *Trichuris* spp. recovered from large intestine of Native lambs sacrificed on day 35 was 2 and from Suffolk sacrificed on 42 was 4.

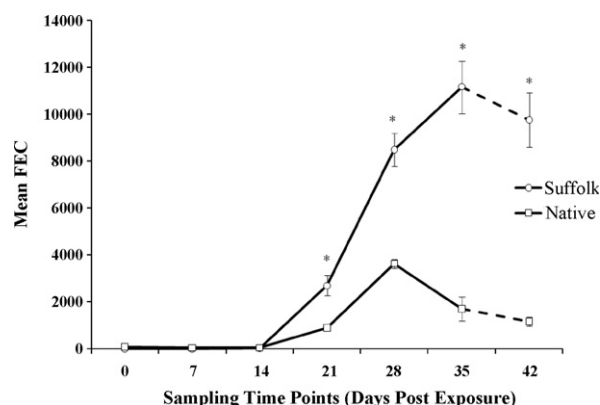


Fig. 1. Mean fecal egg count (FEC) and standard error of the mean in naturally infected Native and Suffolk lambs. Significant differences ($p < 0.05$) are indicated by asterisk marks. Solid line indicates mean for 10 lambs while dashed line indicates mean for five lambs.

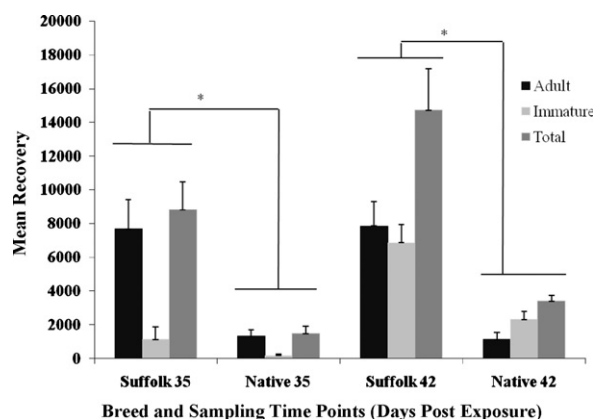


Fig. 2. Mean number of *Haemonchus contortus* recovered from naturally infected Native and Suffolk lambs on days 35 and 42 post-exposures. Significant differences ($p < 0.05$) are indicated by asterisk marks ($n = 5$).

3.2. Systemic parameters

3.2.1. Blood PCV reduction percentage

The starting mean blood PCV in the two groups was significantly different with Native lambs being higher ($p < 0.05$) compared to Suffolk lambs. Therefore, the data were converted into PCV reduction percentages based on the initial PCV values on day 0 of the exposure ((day 0 PCV – sample day PCV/day 0 PCV) $\times$ 100).

The reduction percentage increased at a significantly higher level in Suffolk lambs ($p < 0.05$) (Fig. 3). In Native lambs, the mean reduction percentage was maintained at a lower level between 21 and 35 days post-exposure and recovered to initial stage on day 42 post-exposure. The reduction percentages were significantly higher ($p < 0.05$) in Suffolk lambs after day 21 post-exposure.

3.2.2. Peripheral eosinophil percentage

The Native lambs demonstrated a greater percentage of peripheral eosinophils from days 7 to 42 with two significantly higher ($p < 0.05$) peaks, one each on days 14 and 35 ($p < 0.05$) (Fig. 4). By contrast, Suffolk lamb

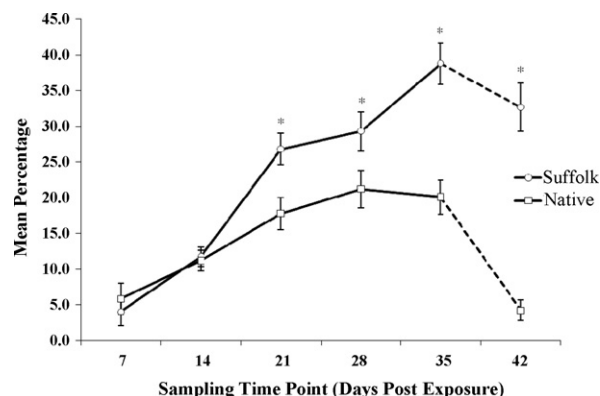


Fig. 3. Mean blood packed cell volume (PCV) reduction percentages and standard error of the mean in naturally infected Native and Suffolk lambs. Significant differences ($p < 0.05$) are indicated by asterisk marks. Solid line indicates mean for 10 lambs while dashed line indicates mean for five lambs.

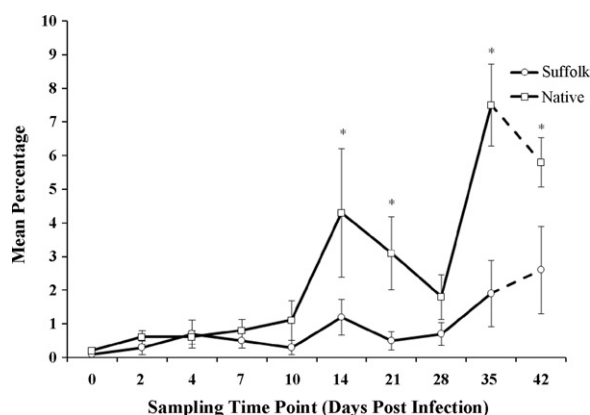


Fig. 4. Mean peripheral eosinophil percentage and standard error of the mean in naturally infected Native and Suffolk lambs. Significant differences ($p < 0.05$) are indicated by asterisk marks. Solid line indicates mean for 10 lambs while dashed line indicates mean for five lambs.

eosinophil percentages remained around the base line with a slight increase after day 28 which continued until day 42 post-exposure.

3.2.3. IL-4, IL-10 and IFN- γ mRNA expression

Native lambs demonstrated a significantly ($p < 0.05$) greater expression of IL-4 mRNA on day 10 post-exposure (Table 2). After day 14 post-exposure, the expression level remained similar for both breeds. However, there was a slight increase for Native lambs after day 28 post-exposure while the Suffolk lambs decreased over the same period.

Suffolk lambs demonstrated a significantly ($p < 0.05$) greater expression of IL-10 on days 7, 10 and 14 post-exposure compared with Native lambs with a tendency to remain higher throughout the experiment (Table 2).

Suffolk lambs demonstrated a trend for greater expression of IFN- γ mRNA compared with Native lambs, however, there was no significant ($p > 0.05$) difference observed between the breeds (Table 2).

3.2.4. Serum immunoglobulin level

No significant difference ($p > 0.05$) in serum level of immunoglobulin isotypes IgM, IgG1, IgG2 and IgA was detected between the two breeds of lambs tested at any sampling time points post-exposure. However, there was a trend for IgG2 in Native lambs to be greater than Suffolk lambs except after day 35 (data not presented) and IgG1 was significantly greater only on day 42 (data not presented) in Native lambs which continually increased in prior sampling time points in Native lambs and remained unchanged in Suffolk lambs.

Serum level of immunoglobulin isotype IgE significantly increased (Fig. 5) ($p < 0.05$) in Native lambs after day 10 and remained at a higher level until day 42 post-exposure. IgE level in Suffolk lambs remained around the base level throughout the experiment.

3.3. Abomasal cellular compositions

The number of eosinophils and mast cells in the mucosa of the cardiac region of the abomasum on day 35 post-exposure was significantly ($p < 0.05$) greater in Native

Table 2

Mean $\pm$ standard error of the mean (SEM) relative quantitative (RQ) values for cytokine mRNAs extracted from peripheral blood in Native and Suffolk lambs at various sampling time points. Significant differences of values between Native and Suffolk lambs ($p < 0.05$) are indicated by asterisk marks ($n = 10$).

Cytokine	Breed	Sampling time points (days post-exposure)								
		0	2	4	7	10	14	21	28	35
IL-4	Native	1.19 $\pm$ 0.18	1.57 $\pm$ 0.30	2.04 $\pm$ 0.71	2.07 $\pm$ 0.93	5.02 $\pm$ 1.55*	0.72 $\pm$ 0.28	0.66 $\pm$ 0.12	0.69 $\pm$ 0.26	1.71 $\pm$ 1.03
	Suffolk	1.18 $\pm$ 0.29	1.59 $\pm$ 0.32	2.84 $\pm$ 1.01	2.80 $\pm$ 1.00	1.09 $\pm$ 0.35	0.82 $\pm$ 0.28	0.48 $\pm$ 0.14	0.96 $\pm$ 0.31	0.45 $\pm$ 0.16
IL-10	Native	0.71 $\pm$ 0.10	0.9 $\pm$ 0.14	2.06 $\pm$ 0.58	1.13 $\pm$ 0.24	2.06 $\pm$ 0.36	1.66 $\pm$ 0.31	3.40 $\pm$ 0.78	5.88 $\pm$ 2.48	5.20 $\pm$ 2.03
	Suffolk	1.42 $\pm$ 0.33	2.04 $\pm$ 0.58	2.94 $\pm$ 0.94	3.17 $\pm$ 0.84*	3.42 $\pm$ 0.82*	3.76 $\pm$ 0.55*	3.30 $\pm$ 1.02	7.39 $\pm$ 2.46	10.79 $\pm$ 8.24
IFN- γ	Native	0.88 $\pm$ 0.11	1.11 $\pm$ 0.14	1.80 $\pm$ 0.39	1.12 $\pm$ 0.14	0.84 $\pm$ 0.20	0.18 $\pm$ 0.10	0.39 $\pm$ 0.06	0.22 $\pm$ 0.08	0.19 $\pm$ 0.05
	Suffolk	1.71 $\pm$ 0.74	1.48 $\pm$ 0.25	2.88 $\pm$ 1.36	1.81 $\pm$ 0.44	0.66 $\pm$ 0.20	0.46 $\pm$ 0.11	0.51 $\pm$ 0.26	0.39 $\pm$ 0.15	0.66 $\pm$ 0.23

compared to Suffolk lambs, whereas neutrophil number was not ($p > 0.05$) (Fig. 6A). A similar trend with a similar magnitude in eosinophil and neutrophil count was observed ($p < 0.05$ for both) on day 42 in both groups of lambs (Fig. 6A). However a greater elevation in mucosal mastocytosis was observed in both groups of lambs on day 42 of infection compared with day 35.

There were significantly ($p < 0.05$) greater numbers of eosinophils, neutrophils and mast cells into the fundic abomasal mucosa in Native compared with Suffolk lambs on day 35 (Fig. 6B). On day 42, however, only the mast cells were significantly ($p < 0.05$) greater, but there was a trend for greater numbers of eosinophils and neutrophils (Fig. 6B). As in the cardiac region, mast cell numbers were greater in both the group of lambs compared with their respective groups on day 35.

A significantly ($p < 0.05$) greater number of neutrophils and mast cells was observed in the pyloric region in the Native lambs compared with Suffolk lambs on day 35 post-exposure (Fig. 6C). Eosinophil count, although not significant ($p > 0.05$), demonstrated a tendency to be in greater numbers in Native lambs. Similarly, mucosal mast cell count was significantly ($p < 0.05$) greater in pyloric region in Native lambs compared with Suffolk lambs on day 42 post-exposure while there was no difference ($p > 0.05$) in neutrophil and eosinophil count although Native numbers were greater (Fig. 6C). As with the cardiac

and fundic regions, mastocytosis on day 42 was greater than on day 35 post-infection in both the groups of lambs.

The globule leukocyte count in the cardiac region of abomasal mucosa was significantly ($p < 0.05$) greater in Native lambs on both days 35 and 42 post-exposure compared with their corresponding Suffolk lambs (Fig. 6A and B). Native lambs demonstrated only a trend for greater counts in the fundic region as the differences were not significant ($p > 0.05$). However, total number of globule leukocytes in the abomasum as a whole was significantly greater ($p < 0.05$) in the Native group of lambs compared with Suffolk lambs. There were no globule leukocytes observed in the pyloric region on either sampling time points.

4. Discussion

Trichostrongyle type eggs were observed in the fecal samples of both the breeds starting 21 days post-exposure to the contaminated pasture. At the peak count on day 35, the Suffolk lambs demonstrated over threefold greater FEC compared with Native lambs. This is in agreement with the results from previous studies comparing Native and Suffolk sheep (Bahirathan et al., 1996; Miller et al., 1998). The decrease in PCV reduction percentage coincided with the decrease in FEC of Native lambs whereas it remained high in Suffolk lambs. When the number of worms recovered from these groups is taken under consideration and correlated with the PCV reduction percentages, it appears that the PCV reduction is directly associated with number of *H. contortus* parasites present in the abomasum. Therefore, greater numbers of *H. contortus* in Suffolk lambs resulted in a greater amount of blood loss in comparison to Native lambs. Whether the reduced worm number in the Native lambs was due to reduced establishment or elimination of established worms is not clear. However, the equivalent blood PCV reduction percentages in both the groups during the initial 2 weeks of the exposure may be an indication of the equivalent level of initial infection in these two breeds. The difference after week 3 post-exposure may be attributable to the difference in establishment or elimination of established worms resulting in the difference in parasite burden observed between the two groups. The reduction in FEC after day 28 post-exposure in Native lambs may be indicative of either elimination of adult worms and or reduced fecundity.

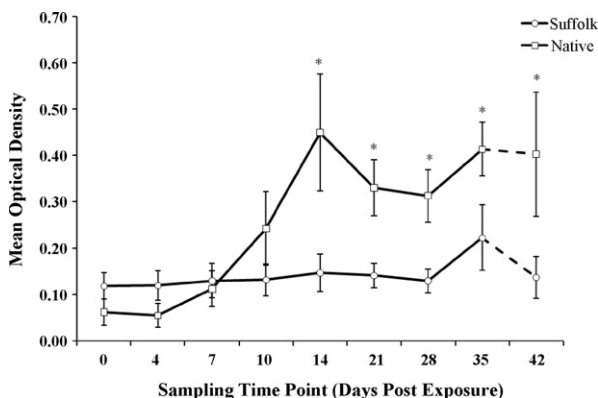


Fig. 5. Mean optical density representing serum level of immunoglobulin isotype IgE in naturally infected Native and Suffolk lambs. Significant ($p < 0.05$) differences are indicated by an asterisk mark. Solid line indicates mean for 10 lambs while broken line indicates mean for five lambs.

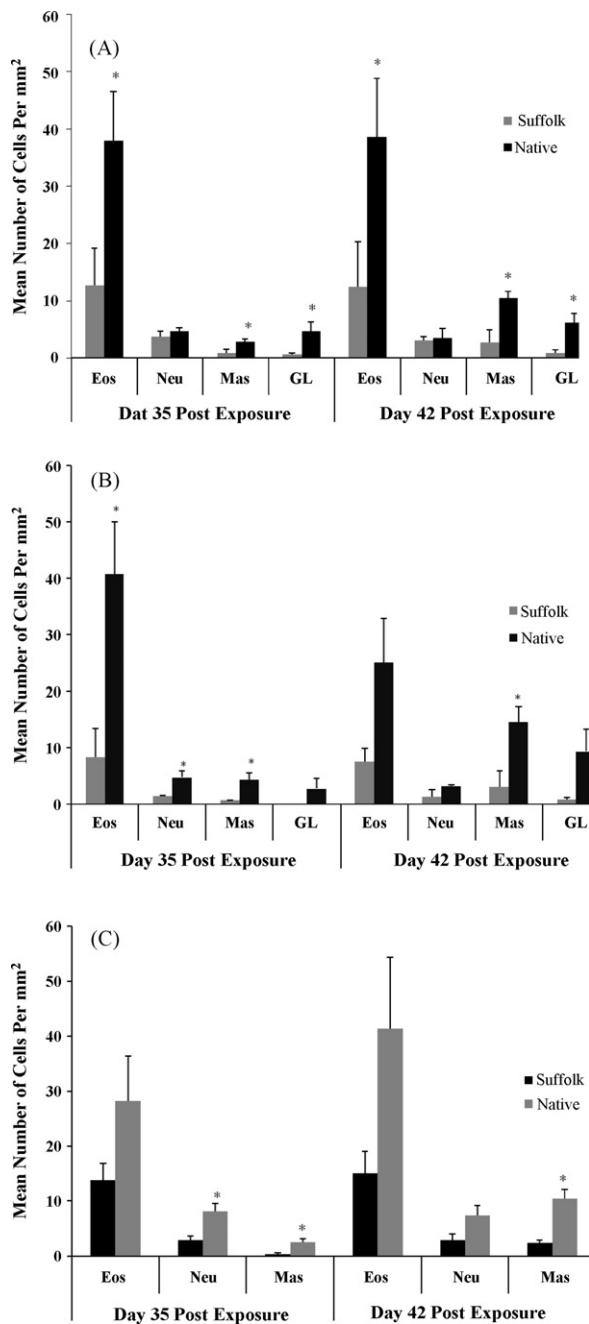


Fig. 6. Mean eosinophil (Eos), neutrophil (Neu), mast cell (Mas) and globule leukocyte (GL) counts in (A) cardiac, (B) fundic and (C) pyloric regions of abomasal mucosa and standard error of the mean in naturally infected Native and Suffolk lambs on days 35 and 42 post-exposure. Significant differences ($p < 0.05$) are indicated by asterisk marks ($n = 5$).

Increased peripheral eosinophil count has been considered to be a potential marker for resistant individuals in Australian lines of Merino and Romney sheep for *H. contortus* and *T. colubriformis*; however a high variability is reported (Douch et al., 1996). Likewise, tissue eosinophilia has been reported in immunized lambs as well as in the resistant lines or breeds of sheep (Gill, 1991; Gill et al., 2000; Woolaston et al., 1996; Hohenhaus et al., 1998;

Hooda et al., 1999). Furthermore, eosinophils have been reported surrounding gastrointestinal larvae in the tissue (Balic et al., 2006; Rainbird et al., 1998). In an *in vitro* study, eosinophils extracted from mammary washes of repeatedly infected sheep were shown to hinder mobility and kill *H. contortus* L3s in presence of anti-L3 surface antibody (Rainbird et al., 1998). Eosinophils and IgE are known to be involved in antibody dependent cell-mediated cytotoxicity. Therefore, greater number of eosinophils and increased IgE may be two of the components involved with greater anti-*Haemonchus* resistance in Native lambs. Our present results also demonstrate a consistent tissue eosinophilia associated with *H. contortus* infection. Balic et al. (2002) reported tissue eosinophilia to occur as early as 3 days post-exposure in secondary infections. Higher eosinophil density in abomasal mucosa is observed in resistant lambs on day 28 post-infection (Gill et al., 2000). The experimental design of the present study did not permit the observation and enumeration of eosinophils in abomasal mucosa during the earlier phase of the infection; however, the results from later time points suggest tissue eosinophilia ensued after the exposure and continued through day 42 post-exposure. Mast cells and globule leukocytes also were found to be associated with resistance to *H. contortus* infection as demonstrated by higher number in Native lambs. Variation in numbers among the different regions may be due to the preferential attachment of the parasites as well as responsiveness of that particular region. *H. contortus* worms have been observed to attach mostly in the pyloric region (data not shown). Why *H. contortus* parasites prefer this particular region for attachment is not understood. Various workers have reported greater mucosal mast cells (MMC) density (Gill et al., 2000) or earlier presence (Lacroux et al., 2006) in resistant lambs compared with random bred lambs and Amarante et al. (1999) reported a negative correlation with *H. contortus* count. Globule leukocytes were observed only in the cardiac and fundic regions of the abomasal mucosa of both the breeds with Native lambs demonstrating significantly greater number. This is in agreement with the findings of other works involving *H. contortus* (Amarante et al., 1999; Lacroux et al., 2006) and in *Oesophagostomum columbianum* (Dobson, 1966) infection in sheep. However, specific location of these cells was not reported in any particular region of the abomasum. Huntley et al. (1992) described globule leukocytes associated with resistance to *H. contortus*. Furthermore, it was also reported that sheep MMC and globule leukocytes contained sheep mast cell protease (SMCP). Tissue concentration of SMCP is directly correlated with the number of MMC present and inversely related to the *Trichostrongylus vitrinus* and *Teladorsagia circumcincta* population present (Huntley et al., 1995). The effector functions of MMC can be brought about in the presence of IgE (Gurish et al., 2004). Upon second exposure, *H. contortus*-sensitized MMC degranulate and release the mediators including SMCP in the mucosal tissue and abomasal mucus of the infected sheep (Huntley et al., 1992). MMC are also regarded as responsible for the leakiness of the epithelium by causing degradation of occludin leading to increased epithelial permeability (McDermott et al., 2003). Increased permeability may

facilitate dissemination of the cells and cellular products in the abomasal lumen resulting in an altered abomasal environment that may be unsuitable for parasite survival. Neutrophils are known to be present in late phase reaction of immediate hypersensitivity although their function is not well understood. They were present in the abomasal mucosa on 35 and 42 days post-exposure.

The most pronounced immunoglobulin response was IgE where Native lambs demonstrated a superior response compared with Suffolk lambs. Kooyman et al. (1997) likewise reported an increased level of IgE, specific to *H. contortus*. Increased level of IgG1 followed by IgG2, IgM and IgA for *H. contortus* infection in sheep was also reported (Schallig et al., 1995). Similarly, resistant lines of sheep were found to produce greater levels of IgE and IgG1 (Gill et al., 2000). On the contrary, these isotypes have been reported to be not significantly correlated with resistance against *H. contortus* (Gomez-Munoz et al., 1999). Lacroix et al. (2006) reported induction of IgA production in secondarily challenged animals by days 7 and 15 in mucus while serum IgG and IgA were detected on 15 and 28 days in response to *H. contortus* excretory/secretory (ES) products, while in *T. colubriformis* infection in resistant lambs, a significantly higher level of IgG1 and IgM were found (Bisset et al., 1996).

In the present experiment, expression of three cytokine mRNAs, IFN- γ , IL-4 and IL-10 was used as representatives Th1, Th2 and regulatory T cell response respectively. IL-4 is the signature cytokine for the Th2 response and is mainly responsible for IgE isotype switching (Matsuda et al., 1995). Upregulation of this cytokine in *H. contortus* infections in sheep has been attributed to increased production of IgE specific to *H. contortus* (Kooyman et al., 1997). The relative IL-4 mRNA expression in the present study suggested an early induction in Suffolk lambs that diminished after day 35 of the experiment. On the other hand, Native lambs had a significantly greater production at day 10 post-exposure which also diminished but later increased towards the end of the experiment. The initial IL-4 expression preceded the first peak of peripheral eosinophils in Native lambs and there is an increasing tendency before the second peak, as well. In addition, peak serum IgE occurred after the peak production of IL-4 mRNA. These results are consistent with IL-4 upregulation for IgE production (Kooyman et al., 1997).

There was no difference in the Th1 type cytokine IFN- γ mRNA expression in the periphery; however Suffolk lambs demonstrated greater expression during the first few days. By day 10, expression decreased and remained around the base level through the rest of the experiment. The expression of mRNA encoding IL-10, a regulatory cytokine, consistently increased from the beginning of the experiment and at times was significantly greater in Suffolk lambs. Expression increased substantially in Suffolk lambs after day 21 of the exposure. The peak in IL-4 mRNA expression in the Native lambs coincided with the decreased level of IFN- γ mRNA on day 10. Similarly, when IL-4 mRNA expression in Suffolk lambs was lowest, IL-10 expression had increased substantially which may be an indication of immunoregulation by IL-10.

It has been reported that abomasal and mesenteric lymph nodes derived cells from *H. contortus* infected lambs had lower expression of IFN- γ compared to uninfected lambs regardless of resistance status while the same line of cells induced an increased expression of IL-5 (Gill et al., 2000). In another study, IL-4, IL-13 and IL-5 expression increased 3 days after challenge, but each with a different pattern. IL-4 expression peaked 5 days post-exposure and remained elevated throughout the challenge period which indicated a role in early or rapid expulsion, while IL-5 and IL-13 expression increased later which indicated a role in the later stages of infection (Meeusen et al., 2005). Similarly, a *T. colubriformis* resistant line of sheep also expressed IL-5 and IL-13 at greater levels compared to a susceptible line (Pernthaner et al., 2005). However, IFN- γ , IL-4 and IL-10 expression was not different between the two lines.

The low expression of IL-4 in both the groups of lambs during third and fourth week of infection may be due to down regulation by the regulatory cytokine IL-10 as it is known to suppress both Th1 and Th2 mediated inflammatory reactions (Helmbly and Grencis, 2003). Down regulation might have resulted in establishment of new incoming larvae that were recovered in greater number on day 42. The depression in expression of IL-4 in both groups indicated that resistant animals are also vulnerable, but they may have some other mechanism to overcome the down regulatory effect. It may be the result of immune modulatory components that are secreted by this parasite. Calreticulin was found to enhance coagulation time and modulate the immune response by binding with C1q (Suchitra and Joshi, 2005) and another 66 kDa adult *H. contortus* ES product which is known to inhibit monocytes (Rathore et al., 2006). The absence of 66 kDa antigen in free living L3 indicated adult worms capable of modulating the immune response. The greater expression of IFN- γ during early infection, especially the peak on day 4 may have been in response to the invasion of larvae in the tissue and the subsequent reduction may have been a result of the shift towards induction of a Th2 type response accompanied by increased IL-4 expression. The constant increased expression IL-10 may account for the reduced expression of IL-4 and IFN- γ and failure to induce production of IgE in Suffolk lambs. The increased expression of IL-10 around day 28 in Suffolk lambs may have accounted for the decreasing trend observed in IgG1 expression and reduced numbers of eosinophils, mast cells, eosinophils and globule leukocytes in the abomasa and peripheral eosinophilia. On the contrary, the reduced expression of IL-10 in Native lambs may not have been sufficient to down regulate the production of immunoglobulins and cellular response.

H. contortus recovery was about six and four times greater in Suffolk lambs compared with Native lambs on days 35 and 42 post-exposure respectively. The immunological responses observed may account for this difference at least in part. The number of immature worms that increased after 35 days may have been an indication of breach in immunity as the peripheral eosinophilia declined during this period.

Taken together, the induction of a Th2 type of immune response characterized by significantly greater eosinophil,

mast cell and globule leukocyte numbers in abomasal mucosa accompanied by increased production of IgE in Native lambs may be the components of the immune response responsible for the reduced establishment and or survival of established *H. contortus* in Native lambs. The relative lack of immune response components, especially IgE in Suffolk lambs is not clearly understood as whether these animals constitutively lack the ability to mount a response or the response is temporarily suppressed due to parasite products. The peripheral eosinophilia and increased serum IgE were associated with the reduced infection in Native lambs. Hence, these might be considered for use as markers while selecting individuals for greater resistance to *H. contortus*.

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