



mRNA profile of Nellore calves after primary infection with *Haemonchus placei*

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ARTICLE INFO

Article history:

Received 26 August 2010

Received in revised form

10 November 2010

Accepted 10 November 2010

Keywords:

Cytokines

Beef cattle

Haemonchus placei

Bos indicus

Gastrointestinal parasites

ABSTRACT

Haemonchus parasites are responsible for many losses in animal production. However, few studies are available, especially of zebu cattle. In this study, we investigated mRNA differences of immune response genes in naïve Nellore calves infected with *Haemonchus placei*, relating these differences to patterns of cellular infiltrate. Calves were infected with 15,000 *H. placei* L3 larvae and after 7 days lymph node and abomasum tissues were collected. IL-2, IL-4, IL-8, IL-12, IL-13, IFN- γ , MCP-1, lysozyme, pepsinogen and TNF- α genes were evaluated by qPCR. Mast cells, eosinophils and globular leukocytes were counted by abomasum histology. In the infected group, IL-4, IL-13 and TNF- α were up-regulated in the abomasal lymph node. In the abomasum, IL-13 increased and TNF- α was down-regulated ($p < 0.05$). No differences were detected for mast cells and eosinophil counts in abomasal tissue ($p > 0.05$). We conclude that for this infection time, there was Th2 polarization but that cellular infiltrate in abomasal tissue takes longer to develop.

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1. Introduction

One of the major constraints of the animal production in the tropical regions is the presence of parasites. Losses in Brazil due to gastrointestinal nematodes are estimated to be about 68 million dollars a year (Honer and Bianchin, 1987; Soutello et al., 2002). In Brazil, *Haemonchus* spp. and *Cooperia* spp. are the most prevalent nematodes (Bianchin et al., 2007; Oliveira et al., 2009).

Brazilian Nellore is a beef breed generally considered resistant to many ectoparasites, but it is not clear if this is true in the specific case of gastrointestinal parasites (Holgado and Cruz, 1994; Oliveira et al., 2009). Although the mechanisms involved in host defense are better understood in *Bos taurus* (Sonstegard and Gasbarre, 2001), less information is available for *Bos indicus* (Bricarello et al., 2007, 2008; Zaros et al., 2010).

Genetic mechanisms underlying the variation of resistance can be related to the development of different profiles of Th1/Th2 cytokines (Meeusen et al., 2005; Huse et al., 2006). The identification of the cytokine genes involved in host response and the explanation of their function should be pursued to establish the different degrees of host resistance, allowing development of better methods of worm control (Gasbarre et al., 2001; Glass et al., 2005).

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It has been shown that Th2 cytokines confer protection to infections caused by endoparasites such as *Trichuris muris*, *H. polygirus*, *Haemonchus* spp., *Nippostrongylus* spp., *Teladorsagia circumcincta* (Else and Finkelman, 1998; Claerebout et al., 2005; Craig et al., 2007; Zaros et al., 2010). Worm resistance in sheep and cattle have been associated with Th2 bias, where higher levels of interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 13 (IL-13) are increased in resistant animals (Gill et al., 2000; Zaros et al., 2010). Craig et al. (2007) found high levels of IL-4 mRNA expression in sheep infected with *T. circumcincta*. Claerebout et al. (2005) found similar results when comparing infected and uninfected cattle exposed to *Ostertagia ostertagi*. IL-4 and IL-13 can increase muscle contractility in the region of infection (Finkelman et al., 2006), and production of IL-5 causes the expulsion of parasites (Bancroft et al., 1998; Garside et al., 2000). Conversely, interferon gamma (IFN- γ), interleukin 2 (IL-2) and interleukin 12 (IL-12) confer susceptibility to that infection (Else and Finkelman, 1998; Zaros et al., 2010).

Histological changes have been described as a consequence of gastrointestinal infections, such as mast cell recruitments, eosinophilia in the mucosa, increase of IgG1, IgG2, IgE, IgA and mucus secretion. Bricarello et al. (2007) studied cattle resistant and susceptible to gastrointestinal parasites, related high levels of IgE and eosinophils with low fecal egg counts. These changes are also related to cytokine polarization, which helps host response (Balic et al., 2002; Meeusen et al., 2005).

In tropical areas such as Brazil, there is great interest in understanding the mechanisms involved in host resistance and the adaptation of breeding stock, such as Nellore cattle, to learn what makes them more resistant to parasite infections. However, the knowledge about this in cattle is restricted to *Bos taurus*. Therefore, the aim of this study was to evaluate mRNA levels of IL-2, IL-4, IL-8, IL-12, IL-13, IFN- γ , monocyte chemoattractant protein 1 (MCP-1), lysozyme, pepsinogen and tumor necrosis factor alpha (TNF- α) genes in the abomasum and abomasal lymph node, as well as to determine the defense cells present in the abomasum of naïve Nellore calves 7 days after primary infection by *Haemonchus placei*.

2. Materials and methods

2.1. Animals

The experiment was carried out in Embrapa Southeast Cattle Station in São Paulo state, Brazil (22°01'S and 47°53'W). Ten Nellore calves descended from 10 cows and three bulls born in November and December of 2005 were used. To keep the animals free from parasite infections, the calves were taken from their mothers immediately after birth and received 2 L of frozen colostrum. They were then kept in individual pens and received 4 L of milk twice a day, along with hay and mineral salt *ad libidum*.

2.2. Calves infection

At 4–5 months of age, the calves were weaned and assigned to two groups of five animals: (1) Infected Group, which was orally infected with around 15,000 L3 *H. placei*

larvae provided by the Embrapa Beef Cattle Station, following the technique described by Roberts and O'Sullivan (Ueno and Gonçalves, 1998) and (2) Control Group, which was kept free from worms during the experimental period. One calf sired by each bull was assigned to each group.

Twice a week samples of feces were collected to determine the EPG (eggs per gram) counts (Ueno and Gonçalves, 1998) to assure that animals had no contact with worms. After 7 days of infection, the calves were sedated with sodium pentobarbital (60 mg/kg of weight) and sacrificed with 2% xylasin cloridrate (1 mL/100 kg of weight).

2.3. Tissue collection

Immediately after slaughter, samples of the abomasum and abomasal lymph nodes were collected and split in two samples: one stored in formalin solution (10%) for histological analysis and the other submerged in liquid nitrogen and stored at -80°C for gene expression analysis.

2.4. Histological analysis

Tissues were fixed in 10% formalin solution for 36 h, washed and stored in 70% ethanol solution and then dehydrated in a series of rising ethanol concentrations, diaphanized with xilol and embedded in paraffin. Histological sections were stained with hematoxylin–eosin (HE) for globule leukocyte and eosinophil counts. Toluidine blue stained sections were used for mast cell counts under an optical microscope. Leukocyte globules were counted under an ultraviolet light microscope.

Cells were counted in 30 random fields of the abomasum surface using a 10 \times eyepiece, with a 100-point grid and 100 \times objective. Cell counts were reported as arithmetic means of cell number/mm² of mucosa.

2.5. RNA extraction and cDNA synthesis

Frozen tissues (-80°C) were macerated in liquid nitrogen and total RNA was isolated using the Trizol[®] (Invitrogen) reagent, following the manufacturer's protocol. RNA concentration and purity were determined by light absorption at 260 nm and OD260:OD280 ratio. Integrity was verified in 1% agarose gel electrophoresis stained with ethidium bromide (20 $\mu\text{g}/\text{mL}$).

Total RNA (5 μg) was used for first-strand cDNA synthesis with the Superscript[™] First-Strand Synthesis System for RT-PCR (Invitrogen), using oligo dT priming, following the manufacturer's protocol.

2.6. Primers information

Primers for IL-2, IL-4, IL-8, IL-12p35, IL-13, MCP-1, TNF- α , RPL-19, GAPDH, lysozyme and pepsinogen genes were described by Zaros et al. (2007), IFN- γ by Coussens and Nobis (2002) and HPRT-1 by Goossens et al. (2005). Amplification efficiencies were obtained by linear regression (efficiency = $10(-1/\text{slope})$), following Pfaffl (2001). Specificities were confirmed in 1% agarose gel and by melting curve analysis (LightCycler, Roche Diagnostics, Mannheim,

Germany) using the program from 70 °C to 95 °C at 0.1 °C/s for all genes studied.

2.7. Real time RT-PCR (qPCR)

Each real time RT-PCR reaction was carried out in 20 μ L of final volume containing 25 ng of cDNA, 0.2 mM of dNTP, 0.1–0.2 μ M of each primer, 2–3.75 mM of MgCl₂, 1.5 units of Taq Platinum DNA polymerase (Invitrogen), 0.04 μ L of Sybr Green 100 \times , 2 μ L of buffer 10 \times 50 mM of KCl, 10 mM of Tris–HCl pH 9.0 (Invitrogen), and 0.5 μ L of dimethyl sulfoxide (DMSO; Sigma). For each sample, the cycle threshold (Ct) mean was obtained and normalized to a reference gene.

Three reference genes were evaluated: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), HPRT-1 (hypoxanthine phosphoribosyltransferase 1) and RPL-19 (ribosomal protein L19).

2.8. Statistical analysis

The relative quantification was evaluated by mathematic modeling based on the PCR efficiencies (E) of the target and endogenous genes and on Ct variation of samples from the experimental groups, according to Pfaffl et al. (2002). For this analysis, the Relative Expression Software Tool (REST[®]) was used, which applies a nonparametric significance test called the Pair Wise Fixed Reallocation Randomisation Test[®].

Histological data (eosinophils, mast cells and globule leukocytes) were analyzed by the GLM procedure using the SAS program (SAS, 2002/2003).

3. Results

3.1. Histological analysis

The average eosinophil and mast cell counts were 30.96 (\pm S.D. 5.55) and 10.31 (\pm S.D. 9) in the non-infected group and 28.41 (\pm S.D. 2.35) and 17.12 (\pm S.D. 1.95) in the infected group, respectively. No significant differences were found between groups for the eosinophil counts ($p=0.30$) and mast cell counts ($p=0.32$) in the mucous membrane of the abomasum (Fig. 1). No globular leukocyte was observed in the slides in any group.

3.2. Relative quantification

Among the three reference genes tested to be used in the relative quantification, the RPL-19 gene was chosen because it presented more constant Ct values ($19 \pm$ S.D. 2.3 in the abomasum and $20.7 \pm$ S.D. 1.2 in lymph node) than the other two genes analyzed.

Relative quantification of target genes showed that IL-4 ($14 \times$; $p=0.002$), IL-13 ($26 \times$; $p=0.003$) and TNF- α ($10 \times$; $p=0.03$) were up-regulated in the abomasal lymph nodes of the infected group in comparison with control group (Fig. 2). In the abomasum tissue, IL-13 was up-regulated ($4.8 \times$; $p=0.03$) in the infected group and TNF- α was down-regulated ($4.0 \times$; $p=0.032$) in the same group (Fig. 3). The mRNA levels of the other genes were not influenced by *H.*

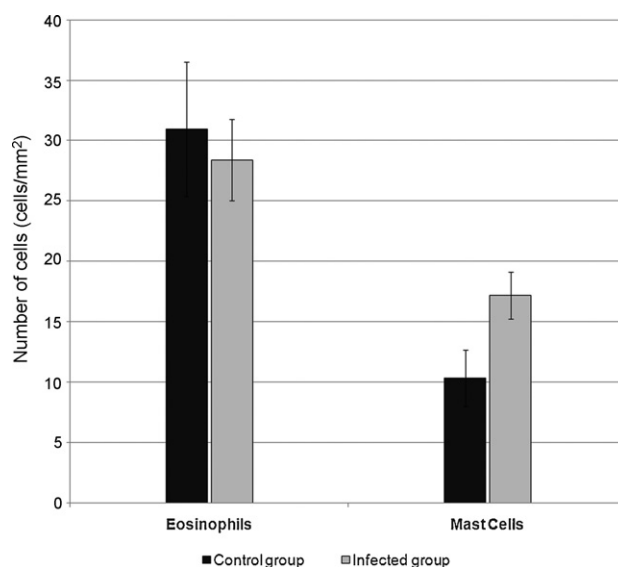


Fig. 1. Mean of eosinophils and mast cells/mm² in the abomasal mucus of control and infected group of Nellore calves with *Haemonchus placei*.

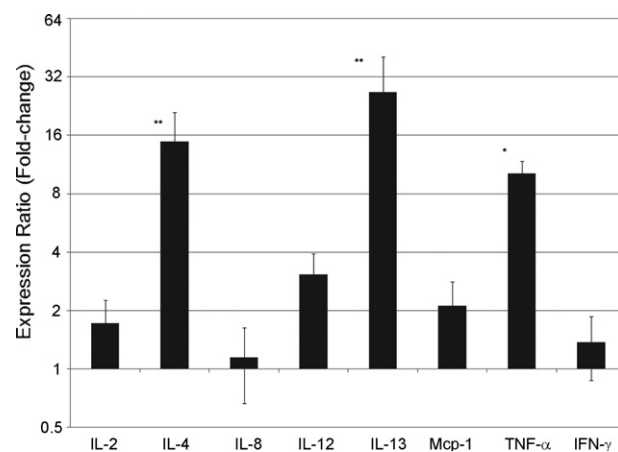


Fig. 2. Ratio of cytokine expression in abomasal lymph node between infected and control group, normalized for reference gene (RPL-19); * $p < 0.05$ and ** $p < 0.01$.

placei larval exposure in the abomasal lymph node, as well as in the abomasum tissue ($p > 0.05$).

4. Discussion

In this study, we compared cytokine gene expression of Nellore calves in primary infection with *H. placei* infections caused by helminths have been studied in many species and have usually been associated with Th2 response in infected animals. In cattle, these infections are not characterized by a severe immune response and most become chronic. Reduction in the number of adult parasites begins after exposure to the parasite and at the same time there is a significant increase in eosinophil, globular leukocyte and mast cell counts in the sites of the infection (Grencis, 2001; Bricarello et al., 2004). Cytokine polarization is indispensable for the correct immune response activation and TCD4+

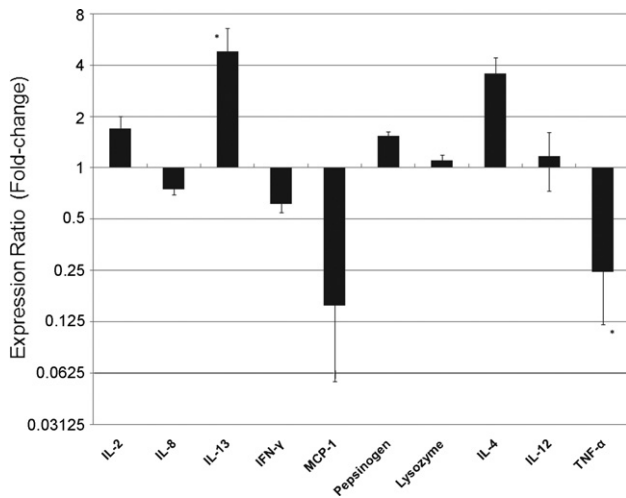


Fig. 3. Ratio of cytokine expression in abomasum between infected and control group, normalized for reference gene (*RPL-19*); * $p < 0.05$.

cells are effectors in *Haemonchus contortus* infections (Gill et al., 1993).

IL-4 and IL-13 are frequently studied because they may be the first genes to have increased levels in response to extracellular parasites, leading to Th2 polarization (Else and Finkelman, 1998) and resistance to animals (Zaros et al., 2010). In this study, the IL-4 mRNA levels in the abomasal lymph node of the infected group were up-regulated 14-fold in comparison to the control group (Fig. 2). Similar results were obtained by (Canals et al., 1997), who observed a significant up-regulation of IL-4 abomasal lymph node of *Bos taurus* cattle infected with *O. ostertagi* on the fourth day post primary infection, increasing gradually until the 28th day of infection. Claerebout et al. (2005) observed an increase in the expression of IL-4 and IL-10 in lymph nodes of immunized calves also infected with *O. ostertagi*, after 3 weeks of infection. In contrast, in the present study we found no difference in this interleukin in the abomasal mucosa, corroborating the results obtained by (Li et al., 2007) and contrasting with those of Lacroux et al. (2006), studying sheep (which are more sensitive to this nematode infection).

IL-13 acts in parasitic infections to promote allergic response, mast cell increase and IgE production, among other reactions. In the present work, severe induction of IL-13 mRNA in abomasal lymph node was observed, about 30 times higher in the infected than in the control group (Fig. 2). In the abomasal mucosa, IL-13 expressed the same pattern, with a fivefold increase in the infected group compared with the control group (Fig. 3). Bancroft et al. (1998), studying knockout mice for IL-13, observed susceptibility to *T. muris* infection as well as a decrease in the response of other Th2 cytokines, inhibiting the expulsion of the parasites. An increase of IL-13 was also observed in sheep immunized and infected primarily with *H. contortus* (Lacroux et al., 2006), showing this cytokine is essential in the protection against gastrointestinal nematode infection.

In fact, some IL-4 and IL-13 functions are redundant, conferring protective response, resistance and expulsion of

the parasites (Else and Finkelman, 1998). We showed that IL-13 had a strong up-regulation, in both tissues, indicating this cytokine could be a precursor of IL-4, stimulating its increase and probably the protective response in the early infection stage in Nellore cattle. This is possible because it has been found that IL-4 starts to increase in the fourth day post-infection of calves with *O. ostertagi* (Canals et al., 1997). There is little data about this polarization in zebu cattle, but it is clear that both cytokines are expressed synergistically and are essential to control this infection. These interleukins act to decrease the fecundity and numbers of helminths by increasing muscular contraction, providing protection by promoting the expulsion of gastrointestinal worms (Else and Finkelman, 1998; Fallon et al., 2002; Lawrence, 2003; Wynn, 2003). In similar conditions, primarily infected *Bos taurus* cattle and sheep (Claerebout et al., 2005; Lacroux et al., 2006), as well as resistant Nellore cattle (Zaros et al., 2010), had high levels of these cytokines.

TNF- α is a pro-inflammatory factor that may have an important role in gastrointestinal infections. Hayes et al. (2007) observed that TNF- α acts to increase both cytokines Th1 (IFN- γ) and Th2 (IL-13). Although this pattern is not well established yet, functions related to Th2 polarization have been reported (Artis et al., 1999). Some works concluded that this pro-inflammatory factor is associated to resistance in sheep (Pernthaner et al., 2005) and cattle (Li et al., 2007). In our work, TNF- α was about eight times higher in the lymph node (Fig. 2) and fourfold less expressed in the abomasal mucosa (Fig. 3) of the infected animals compared to the uninfected ones. A contrasting pattern was observed in both tissues studied. The presence of TNF- α was characterized to potentialize the expulsion of parasites by IL-13, conferring protection in the host (Artis et al., 1999), as well as, it was correlated to induction of host resistance in early larval stages (Babu and Nutman, 2004). So, differences of TNF- α found in the two tissues studied could be a result of tissue collection when the immune response started to be established and when changes in larval stages were still ongoing. Then, although, at this time, this cytokine could be helping the Th2 polarization in the lymph nodes, the presence of parasitic secretions in the abomasum could exert some local immunomodulation. It is known that the *Haemonchus* spp. L4 larvae stage is capable of inducing changes in the host immune profile to evade host response (Allen and MacDonald, 1998). As in early infection stages, TNF- α has been reported to promote parasite expulsion, this molecule could be a target for immunomodulation by the parasite (Maizels and Yazdanbakhsh, 2003). TNF- α down-regulation may be caused by mast cell inhibition and may turn resistant animals in susceptible (Behnke et al., 2003; Pernthaner et al., 2005). Therefore, maintaining low TNF- α level in the host would be beneficial for completion of parasitic life cycle. Artis et al. (1999) found that low TNF- α level delays the expulsion of parasites from the host and that IL-4 and IL-13 levels remain up-regulated, as observed in this work.

During gastrointestinal infections, increases of mast cells and eosinophils are usually observed (Gasbarre, 1997; Else, 2005). In sheep, these cells are involved in rejection of *H. contortus*. Eosinophils are recruited to the abomasum of sheep during primary infection (Balic et al., 2000, 2002)

and are related to death of the parasite (Balic et al., 2006). In cattle, higher numbers of mast cells and eosinophils were observed in resistant compared to susceptible *Bos indicus* (Zaros et al., 2010). However, no differences between infected and control groups were observed for these cells in the present trial (Fig. 1). This might be due to the short period of infection, only 7 days. In cattle infected with *O. ostertagi* it is known that cells accumulate after adult worms are present for 1 or 2 days in the abomasum (Scott et al., 1998; Simpson, 2000). In the present work, infection was caused by larvae and abomasums were obtained 7 days after infection, a period when most of the larvae should be in L4 stage, which marks the beginning of the hematophagous phase and is expected to stimulate host protective response. From our results, it was demonstrated that, in animals that had not been exposed to *H. placei* beforehand, this period of time was not enough to deflagrate local response in the mucosa.

For the other genes evaluated (IL-2, IL-8, IL-12, IFN- γ and MCP-1), as well as for pepsinogen and lysozyme no difference in mRNA profile was observed in any of the tissues studied. As in this work, infections with *H. placei*, *Cooperia* spp. and *Ostertagia* spp. were found not to be associated with changes in the levels of IL-8 (Li et al., 2007; Bricarello et al., 2008; Zaros et al., 2010). Nevertheless, variations were reported for some of these genes, as in the work by Li et al. (2007), which concluded that IL-2, IFN- γ and IL-12 were responsible for conferring resistance to Angus heifers infected with *O. ostertagi*. Other evidence for the involvement of these genes in response to parasite has been found in Nellore cattle infected with *H. placei*, where a strong Th2 profile was detected in resistant animals (Zaros et al., 2010).

In conclusion, we suggest that IL-4 and IL-13 initiate the immune response in the abomasal lymph nodes and abomasal mucosa in the first infection of naïve Nellore calves with *H. placei*, evidencing a possible initial Th2 polarization. It may be possible that TNF- α helps in this polarization and that this cytokine is modulated by the parasite. In contrast, no significant increase in eosinophils and mast cells was detected, indicating that the local inflammatory response to *H. placei* occurs later during the infection of *Bos indicus* Nellore calves.

Acknowledgments

We thank Dr. Ivo Bianchin, of the Embrapa Beef Cattle Research Center, for providing the larvae and Dr. Rogério Taveira Barbosa and Dr. Rui Machado for taking care of calves. This work was financially supported by Prodatab and Embrapa. We thank CAPES for providing scholarships to AMGI and LCN and CNPq for fellowships to LLC and LCAR.

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