Received: 7 March 2016 | Accepted: 23 March 2017

DOI: 10.1111/pim.12427

BRIEF DEFINITIVE REPORT

WILEY Parasite Immunology

Cytokine and antioxidant gene profiles from peripheral blood mononuclear cells of Pelibuey lambs after *Haemonchus contortus* infection

Z. Estrada-Reyes[1] | M. E. López-Arellano[2] | F. Torres-Acosta[1] | A. López-Reyes[3] | A. Lagunas-Martínez[4] | P. Mendoza-de-Gives[2] | R. González-Garduño[5] | S. Olazarán-Jenkins[2] | D. Reyes-Guerrero[2] | G. Ramírez-Vargas[2]

[1]Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán, Mérida-Xmatkuil, Yucatán, México

[2]Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria, INIFAP, Jiutepec, Morelos, México

[3]Instituto Nacional de Rehabilitación, Tlalpan, Ciudad de México, México

[4]Instituto Nacional de Salud Pública, Cuernavaca, Morelos, México

[5]Unidad Regional Universitaria Sur-Sureste, Universidad Autónoma de Chapingo, Tabasco, México

Correspondence
Ma. Eugenia López-Arellano, Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria, INIFAP, Jiutepec, Morelos, Mexico.
Email: mlopez.arellano@gmail.com

Funding information
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), México; Consejo Nacional de Ciencias y Tecnología, CONACYT

Summary

The expression profiles of cytokines and antioxidant genes were determined from an experimental infection with *H. contortus* in Pelibuey lambs. The infection was followed for 34 days (d) to determine the number of eggs per gram (epg) and the packed cell volume (PCV). Differential white cell counts and expression profile estimations of *IL-2*, *IL-4*, *IL-5*, *IL-6*, *IL-8*, *IL-10*, *IL-13*, *FCεR1A*, *GPX* and *SOD1* were determined at 0 hour, 4 hours, 2 days and 14 days post-infection (PI) in infected and control groups. Comparison of the fold change between 0 and 4-hours, 4-hours and 2-days and 2- and 14-days periods was performed. Significant differences ($P<.05$) between epg (>2000) and PCV (>30%) were determined after 21 days and were also observed with regard to monocyte and lymphocyte cells after 2 and 7 days PI. At 0 hour and 14 days PI, the *GPX* and *IL-2* genes showed a 0.37- and 0.49-fold decrease in expression, respectively. In contrast, upregulation was observed at 4 hours of *IL-8* (2.58) and *FCεR1A* (2.71), at 2 days for *IL-4* (2.14) and *IL-8* (4.02) and at 14 days for *IL-2* (0.41), *IL-10* (2.35) and *FCεR1A* (2.28). The comparison between the intervals of infection showed high expression values against *H. contortus* infection in Pelibuey sheep after the 2nd period of PI involving a dichotomy T cells.

KEYWORDS
Cytokines, expression profiling, *Haemonchus contortus*, immunoregulation, peripheral blood mononuclear cells, ruminant

1 | INTRODUCTION

Haemonchus contortus is one of the most important gastrointestinal nematodes (GIN) affecting small ruminants mainly in tropical regions of the world. The haemonchosis is able to induce the production of T_H1 (eg, IL-2 and IL-8) and T_H2 (eg, IL-4, IL-5, IL-6, IL-10 and IL-13) cytokines; either in the peripheral blood or in the abomasum wall[1,2]. However, the level of the host immune response against GIN may be variable. The development of a mature immunity against GIN depends on different factors, such as breed, age and nutrition[3]. The high prevalence of *H. contortus* is the main animal-health problem in sheep-meat producers in several tropical regions of different countries, such as Mexico[2,3]. Some studies have proposed the meat-sheep Pelibuey as a possible resistance breed against *H. contortus* according to its reduced faecal egg count (FEC)[2,4]. Recently, the importance of IFN-γ (T_H1) and IL-5 and IL-6 (T_H2) cytokines has been demonstrated to identify low and high Pelibuey lambs naturally infected with *H. contortus*[2,5]. However, the identification and selection of a resistance host are not easy because of the variable response of the host against nematodes, it requires more understanding of the interaction between the

immune response and infected lambs with *H. contortus*. The aim of this study was to analyse the expression of T_H1 and T_H2 cytokines, immunoglobulin E receptor (*FCεR1A*) and oxidant factors *GPX* and *SOD1* in peripheral blood mononuclear cells (PBMCs) in inoculated Pelibuey lambs with *H. contortus* at 0 hour, 4 hours, 2 days and 14 days post-infection (PI).

2 | MATERIALS AND METHODS

This study was conducted at CENID PAVET, INIFAP, Mor., México. Eleven Pelibuey lambs were obtained from a sheep flock free of GIN maintained in the CENID. The ewes were kept in pens with concrete floors for more than 3 years. After birth, the lambs were allowed to suckle during the day for 3 months. The lambs were then separated from their mothers and kept in a shed to begin the weaning process. Before the study, the lambs were treated with levamisole (7.5 mg/kg of live weight, administered intramuscularly).

2.1 | Animal welfare

All experimental sheep were treated following the Norma Oficial Mexicana (Official Rule Number) NOM-051-Z00-1995 (www.senasica.gob.mx) as well the Ley Federal of Sanidad Animal Federal Law for Animal Health (www.diputados.gob.mx/LeyesBiblio/ref/lfsa.htm).

2.2 | *Haemonchus contortus* lamb artificial infection protocol

Three nematode-free lambs served as the controls. Eight lambs orally received 350 L_3 of *H. contortus* per kg of body weight on day 0. The *H. contortus* isolate was obtained from a Pelibuey sheep with monospecific infection. Infective larvae (L_3) were obtained from faecal cultures maintained at 26°C for 7 days, and larvae were harvested using the Baermann technique[6].

2.3 | Blood and faecal samples

Blood samples were collected at 0 hour, 4 hours, 2 days and 14 days PI via intravenous puncture into vacutainer EDTA tubes. Samples were processed to separate PBMCs from control and experimental groups, using a mixture with PBS pH 7.2 (1:1) layered onto a Ficoll-Paque TM PLUS (GE, Healthcare, Sweden). An aliquot of each blood sample was used to determine the percentage of PCV using the microhaematocrit technique, and differential white cell count (%) was also performed. Faeces were individually obtained (30 g) from lambs on 0, 14, 21, 24, 27 and 34 days PI to determine the number of epg using the McMaster technique with saturated solution[6].

2.4 | PCR Array and synthesis

Primers used for the Custom RT^2 Profiler PCR Array Data Analysis (Cat. Num. CAPB11185R) were designed and synthetized by Qiagen (Hilden, Germany). The array consisted of 100 PCR tubes of 0.2 mL per array. A total of seven cytokines, two oxidative radicals and immunoglobulin genes were evaluated per sample; *gapdh* was used as the reference gene. The RNA extraction and reverse transcription (RT-qPCR) were performed with Qiagen (Hilden, Germany) products following the manufacturer's instruction. Total RNA was isolated from lymphocyte samples using QIAzol Lysis reagent and suspended in 30 μL of nuclease-free water. The yield was calculated from a spectrophotometric measurement at 260 nm (NanoDrop-1000, Thermo Scientific), and the purity was assessed using a range of 260-280 nm. Total RNA was reverse-transcribed into cDNA using a RT^2 First Strand Kit. Forty cycles of amplification, with an initial incubation at 95°C for 10 minutes, followed by denaturation at 95°C for 15 seconds and annealing at 60°C for 30 seconds, were performed for the RT-qPCR assays.

2.5 | Statistical analysis

All general linear model (GLM) and *t* test procedures were analysed using SAS version 9.0 (Cary, NC, USA, 2002). Differences were considered statistically significant when $P<.05$ for epg, PCV and white cells. The results of epg and PCV analysed by GLM were compared using a *t* test. The white cells analysis was carried out with raw data using repeated measures. This analysis was performed using fixed effects collected from trait periods and counting cells. For the relative quantification of gene expression, the comparative C_T method was used from each sample[7]. Differences between C_T values from *gapdh* and each target gene (defined as ΔC_T) were analysed from infected and control groups at 0 hour, 4 hours, 2 days and 14 days PI. The relative fold change increases or decreases were then calculated as $2^{\Delta\Delta CT}$ and were used to determine the fold change and *P*-values between three intervals of infection defined as 0 hour-4 hours, 4 hours-2 days and 2 days-14 days. Differences were considered statistically significant when $P<.10$ and $P<.20$.

3 | RESULTS

3.1 | Phenotypic traits

Analysis between epg and the PCV showed significant differences ($P=.05$) through all experimental periods. The epg values in faeces of infected animals were identified after 21 days prepatent period and ranged from 2281±2082 to 4936±5212. The average of PCV for the infected group was 38±2.79 and 33±2.63 for the control group. There were no significant differences ($P>.05$) in the number of basophils, neutrophils and eosinophils. In contrast, significant differences ($P<.05$) were observed for the monocytes at 2nd (16±8) and 7th (10.12±4.8) days, with decreased response in the infected group compared with control group (20.5±4.3 and 18.5±6.4). Unlike the monocyte response, the number of lymphocytes increased at 7 days PI (77±16) compared with the control group (33.5±6.3).

3.2 | Relative expression level of interleukins and oxidative genes

Important differences were observed in the fold change of interleukin immunoregulatory-related genes *IL-5*, *IL-6*, *IL-8*, *SOD1* and *GPX* in the average samples at 0 hour, 4 hours, 2 days and 14 days ($P<.05$). Unlike the average samples, large variations of expression were observed in each infected host ($P>.2$). However, increased expression was determined for the *IL-8* expression that reached its maximum (4.02-fold at 2 days) after the 2nd day PI. There was approximately a 2.0-fold increase in the average expression level across the *IL-2* (2.0-fold at 14 days), *IL-4* (2.14-fold at 2 days), *IL-8* (2.58-fold at 4 hours), *IL-10* (2.35-fold at 14 days) and *FCεRIA* (2.29-fold at 14 days) PI compared to the control group and internal gene expression. In contrast, downregulation was demonstrated in the genes *GPX* (0.37-fold at 0 hour and 0.49-fold at 14 days) and *IL-2* (0.41-fold at 4 hours).

3.3 | Comparative expression levels form three infection periods

Infection of *H. contortus* showed large changes in gene expression through three different periods of time (Table 1 and Figure 1). The first period at 0 and 4 hours PI showed high expression of *IL-8* (1.04-fold; $P<.2$) and *SOD1* (1.25-fold; $P<.2$). However, the highest gene expression corresponded to the second period of infection at 4 hours and 2 days PI. In this period, *IL-2* had the highest level of expression (4.31-fold; $P<.15$), followed by *IL-10* (2.87-fold; $P<.12$), *FCεRIA* (2.04-fold; $P<.16$) and *IL-4* (1.99-fold; $P<.08$). Other cytokines and stress factors also showed high expression, but a large variability was observed from the infected group (between $P<.3$ and $P<.4$). During the last period between 2 and 14 days PI, the expression level was decreased and reached the maximum level for *IL-10* (0.76-fold; $P<.2$), *IL-2* (0.58-fold; $P<.15$), *GPX* (0.41-fold; $P<.14$), *SOD1* (0.38-fold; $P<.15$), *IL-4* (0.34-fold; $P<.09$) and *IL-5* (0.35-fold; $P<.12$).

4 | DISCUSSION

In the present study, the expression profile of specific cytokines and antioxidant molecules commonly associated with *H. contortus* infection was evaluated in the hair Pelibuey lambs. Three windows of time were used: 4 hours PI, when the *H. contortus* L_3 started the infection; day 2 PI, when the infection was established; and day 14 PI, before established infections became patent. As infections with *H. contortus* escalated, the number of epg increased, reaching a maximum level on the 24th day PI. In contrast, a similar PCV level (~33%) was observed during all experimental periods, indicating a tolerance to the nematode infection. The beginning of infection is an aggressive process that causes stress, releasing some oxidative radicals and activating immune cells associated with the host response against nematodes[8,9]. Through infection, the larvae development, feeding habit and surface and secreted products are involved in the invasion of host tissues (abomasal and peripheral blood), constituting the first contact with the immune system[1,2]. The invasive process causes attraction of different immune cells, some of which are promoted by secreted products released through the peripheral blood cell, for example, polymorphonuclear and T cells[9-11]. However, important changes of expression level may occur. For instance, a positive association between oxidative molecules and hyperimmunized *H. contortus* hosts was observed[9]. In contrast, Pelibuey lambs displayed decreased oxidative *GPX* gene expression through an early host response against *H. contortus*. Changes in the host response involve other factors such as age, nutritional status and course of infection[3]. Based on these considerations, our work aim was focused before the infection become patent. Therefore, comparison between different intervals of *H. contortus* infection

TABLE 1 Relative quantification of mRNA transcripts in polymorphonuclear blood cells between Three different periods of *H. contortus* infection in Pelibuey lambs. Values of these lambs are averages of measurements taken from the fold change. Periods of time are 0 h=Pre-infection, 4 h, 2 and 14 d=days post-infection. *IL*=interleukins, *SOD1*=superoxide dismutase 1; *GPX*=glutathione peroxidase; *FCε*R1A=IgE receptor; *gapdh*=glyceraldehyde-3-phosphate dehydrogenase

Periods of time						
	0-4 h		4 h-2 d		2-14 d	
	Fold change	*T* test	Fold change	*T* test	Fold change	*T* test
Gene	$2^{(-\Delta\Delta Ct)}$+SE	*P*-value	$2^{(-\Delta\Delta Ct)}$	*P*-value	$2^{(-\Delta\Delta Ct)}$	*P*-value
IL-2	0.31	.40	4.31	.15[b]	0.58	.15
IL-4	0.56	.15[b]	1.99	.08[a]	0.34	.09[a]
IL-5	0.86	.36	1.07	.49	0.35	.12[b]
IL-6	0.48	.26	2.29	.40	0.24	.13[b]
IL-8	1.04	.21	1.74	.38	0.25	.15[b]
IL-10	0.24	.29	2.87	.12[b]	0.76	.21
IL-13	0.71	.28	1.51	.46	0.29	.13[b]
SOD1	1.25	.21	1.08	.41	0.38	.15[b]
GPX	0.78	.26	1.22	.31	0.41	.14[b]
FCεRIA	0.53	.43	2.04	.16[b]	0.32	.10[a]
gapdh	1	0	1	.00	1	0

Different letters are [a]$P<.10$; [b]$P<.2$

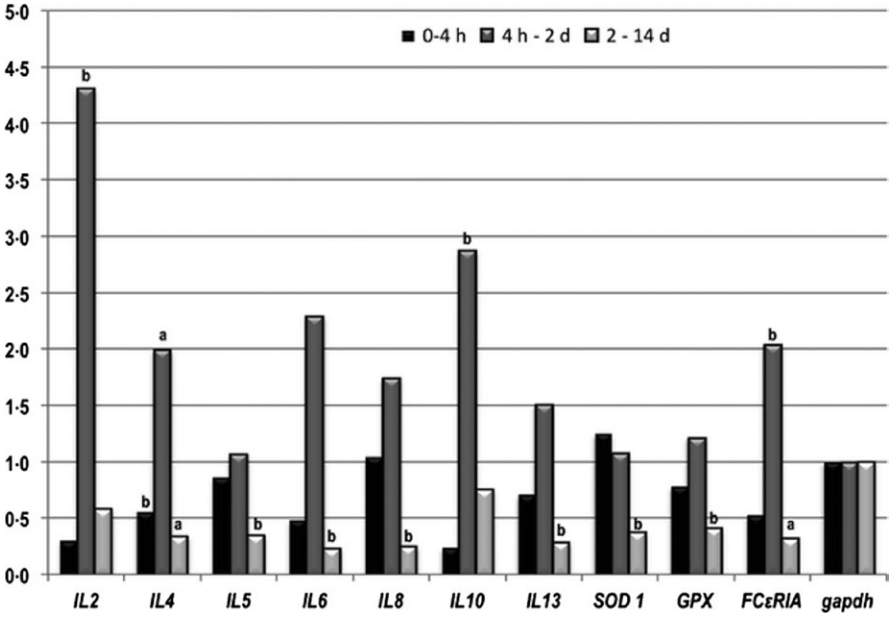

FIGURE 1 Immune factors transcription in polymorphonuclear cells in Pelibuey lambs infected with *H. contortus* infection. Values of these lambs are averages of measurements taken from the fold change (comparison between periods). The three experimental periods corresponded at 0 h=Pre-infection, 4 h, 2 and 14 d=days post-infection. Different letters in bold show *P*-values of: a=*P*<.10; b=*P*<.2

displayed a strong early expression of T_H1 and T_H2 cells at 4 hours-2 days PI, reaching the highest level for *IL-2*, followed by *IL-6*, *IL-8*, *IL-10*, *IL-4* and *FCεR1A*, involved in the regulation of the nematode infection[3,12]. In contrast, a moderate level of expression was observed at 0 hour-4 hours and 2 days-14 days periods, showing less changes of expression during the last period, probably caused by the mature immune response. Most of these molecules were expressed after the 2nd period of infection where *H. contortus* progresses to the endoparasitic stage, where monocytes and lymphocytes are responsible for presenting the peptide antigens produced by nematodes. Because monocytes are known to modulate pro-inflammatory signals (eg, *ILs 8 and 10*), this result suggests that monocytes are involved in the early phase of haemonchosis. The *FCεRIA* and *IL-8* keep a close immunological association with *IL-4* gene function to promote isotype switching of murine B cells to IgE through eosinophils and neutrophils[13]. The upregulation of T_H1 and T_H2 cells and oxidative factors between 4 hours and 2 days PI might indicate the activity of antigenic products through peripheral blood cells; however, more studies are required.

5 | CONCLUSIONS

The present study demonstrated that primary infection with *H. contortus* induced early expression of T_H1 and T_H2 cytokines and innate factors in the peripheral blood of Pelibuey lambs. These cytokines and innate factors were associated with early infection periods and could be essential to establish a mature immune response in Pelibuey lambs in tropical areas.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), México. Miss Z.M. Estrada-Reyes was partially supported by the Consejo Nacional de Ciencias y Tecnología, CONACYT. The authors gratefully acknowledge Red Temática Farmocoquímicos (CONACYT) for kindly support the English edition.

COMPETING INTERESTS

The authors declare that they no competing interests.

AUTHOR CONTRIBUTIONS

Estrada-Reyes Z MSc and López-Arellano ME performed the research, analysed data from each experimental assay and wrote the present manuscript. Torres-Acosta F, Olazarán-Jenkins S carried out the parasitological and haematological assays. González-Garduño R carried out the interpretation and statistical analysis. Ramírez-Vargas G and Reyes-Guerrero D contributed to the bioinformatics and molecular studies. López-Reyes A and Lagunas-Martínez designed and helped to analyse the molecular studies. Mendoza-de-Give P contributed with essential reagents and conceived and participated in the drafted manuscript.

DISCLOSURE

The authors of this manuscript have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

REFERENCES

1. Gossner A, Wilkie H, Joshi A, Hopkind J. Exploring the abomasal node transcriptome for genes associated with resistance to the sheep nematode *Teladorsargia circumcincta*. *Vet Res*. 2013; 44:68.

2. Estrada-Reyes ZM, López-Reyes AG, Lagunas-Martínez A, et al. Relative expression analysis of *IL-5* and *IL-6* genes in the tropical sheep breed Pelibuey infected with *Haemonchus contortus*. *Parasite Immunol*. 2015;37:446-452.
3. Greer AW, Hamie JC. Relative maturity and development of immunity to gastrointestinal nematodes in sheep: an overlooked paradigm? *Parasite Immunol*. 2016;38:263-272.
4. González JL, López-Arellano ME, Olazarán-Jenkins S, et al. Phenotype characterization of Pelibuey native lambs to *Haemonchus contortus* infection. *Ann N Y Acad Sci*. 2008;1149:164-166.
5. Reyes-Guerrero DE, López-Arellano ME, González-Garduño R, et al. Identification of B allele of interferon gamma gene associated to *Haemonchus contortus* resistance infection in Pelibuey lambs. *Quehacer Científico* 2016;11:3-9.
6. Liébano-Hernández E. *Manual de Diagnóstico Para la Identificación de Larvas Infectantes de Nematodos Gastrointestinales en Rumiantes*. Morelos, Mexico:Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias;2011;1:27-30.
7. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29:2002-2007.
8. Ibelli AMG, Nakata LC, Andréo R, et al. mRNA profile of Nellore calves after primary infection with *Haemonchus placei*. *Vet Parasitol*. 2011;176:195-200.
9. Lees MS, Robinson NA, Ingham AB, Kotze AC, Piedrafita DM. Dual oxidase 2 and glutathione peroxidase gene expression are elevated in hyperimmunised sheep challenged with *Haemonchus contortus*. *Vet Parasitol*. 2011;179:113-122.
10. Balic A, Bowles VM, Meeusen ENT. Cellular profiles in the abomasal mucosa and lymph node during primary infection with *Haemonchus contortus* in sheep. *Vet Immunol Immunopathol*. 2000;75:109-120.
11. Menzies M, Reverter A, Andronicos N, Hunt P, Windon R, Ingham A. Nematode challenge induces differential expression of oxidant, antioxidant and mucous genes down the longitudinal axis of the sheep gut. *Parasite Immunol*. 2010;32:36-46.
12. MacKinnon KM, Bowdridge SA, Kanevsky-Mullarky I, Zajac AM, Notter DR. Gene expression profiles of hair and wool sheep reveal importance of Th2 immune mechanisms for increased resistance to *Haemonchus contortus*. *J Anim Sci*. 2015;93:2074-2082.
13. Zaros LG, Bricarello PA, Amarante AFT, et al. Cytokine gene expression in response to *Haemonchus placei* infections in Nelore cattle. *Vet Parasitol*. 2010;171:68-73.

How to cite this article: Estrada-Reyes Z, López-Arellano ME, Torres-Acosta F, et al. Cytokine and antioxidant gene profiles from peripheral blood mononuclear cells of Pelibuey lambs after *Haemonchus contortus* infection. *Parasite Immunol*. 2017;39:e12427. https://doi.org/10.1111/pim.12427