Differential IL10 mRNA Profiles Associated to \textit{Babesia bovis} and \textit{B. bigemina} Infection Levels in Persistently Infected Animals

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Abstract

This work aimed to find quantitative phenotypic traits that can be used to discriminate the levels of resistance/susceptibility to \textit{B. bovis} and \textit{B. bigemina} in two groups of cattle presenting the highest (H) or lowest (L) infection levels and \textit{Rhipicephalus microplus} ticks count. The animals were selected from a previous study of 50 Canchim (5/8 Charolais/zebu) heifers raised in an endemic area for these parasites. These animals were evaluated regarding their TNFα, IL10, IFN-γ, IL12 and iNOS mRNA levels. No differences were found between these groups regarding TNFα, IFN-γ, IL12β or iNOS transcripts. However, the IL10 transcripts were significantly higher in the H group compared to the L group. Moreover, significant correlation coefficients were observed between \textit{B. bovis} loads and both IL10 and IFN-γ transcripts, while no correlations were found for \textit{B. bigemina} loads and all tested immune-related transcripts, suggesting that differential IL10 mRNA profiles were closely associated to \textit{B. bovis} loads. Our results have contributed to a better understanding of the immune responses against \textit{Babesia} infection, as we demonstrated that the IL10 cytokine levels might also influence or be influenced by parasitemia levels in persistently infected animals.

Keywords

\textit{Babesia bovis}, Cytokines, Gene Expression, IL10, RT-qPCR

1. Introduction

\textit{Babesia bovis} and \textit{B. bigemina} cause bovine babesiosis and lead to significant
losses for cattle producers in extensive tropical areas of the world [1] [2]. The transmission rate of *B. bigemina* by ticks is higher than *B. bovis*, making its prevalence higher in herds where the disease is endemic [1]. On the other hand, pathogenesis induced by *B. bovis* infection is considered the most severe among illnesses produced by babesial parasites, characterized by sequestration of infected erythrocytes to microcapillary endothelia of vital organs and a hypotensive shock syndrome, leading to central nervous system damage, severe sequels or mortality, especially in adult naïve animals [3] [4] [5]. This severe pathogenesis is thought to be induced by overproduction of some cytokines (“cytokine storm”), as IFN-γ and TNFα, in addition to other soluble mediators, including nitric oxide (NO), which are usually associated with protective immune responses against intracellular pathogens [4] [6]. Protective immune mechanisms involved in the resolution of acute infection in immunologically naïve animals infected with virulent *B. bovis* parasites seem to be dependent on strong innate responses which leads to mononuclear phagocytes activation by IFN-γ and parasite-derived products, resulting in phagocytosis and NO production. While, in persistently infected animals which have controlled parasitemia, or in successfully immunized animals, antigen-specific CD4+ T cells are central to the adaptive response through production of IFN-γ [4]. High levels of IL10 mRNA transcripts were previously associated to increased severity of clinical disease by *B. bovis* infection due to a dampening effect on the immune response in general, especially by downregulating IFN-γ and iNOS genes [7]. Moreover, in a comparative study of calves and adults experimentally infected with the T2Bo isolate of *B. bovis*, differential cytokine profiles were observed according to different age groups [8]. These authors reported that IL10 expression levels were higher and remained prominent longer in adults, which as expected presented severe disease, while in calves, early induction of IL12 and IFN-γ were associated with protection, and preceded IL10 production.

Differences in the susceptibility to hemoparasites have been verified within breeds, suggesting that it may be possible to select resistant phenotypes [7] [9] [10] [11]. The present study evaluated the TNFα, IL10, IFN-γ, IL12β, and iNOS transcripts levels in persistently infected animals presenting high or low levels of *B. bovis* and *B. bigemina* loads, aiming to elucidate better the immune mechanisms involved on babesial clearance.

### 2. Material and Methods

#### 2.1. Animals and Experimental Area

Experimental study was performed using 50 females of Canchim breed (5/8 Charolais/Zebu), weaned between 8 to 10 months of age, and raised in pasture covered by *Panicum maximum*, at the experimental farm of EmbrapaPecuária-Sudeste—São Carlos, SP—Brazil (22°0’55”S, 47°53’28”W). Animals received water and mineral salt mixture *ad libitum*. This region was identified to be endemic for the occurrence of *R. microplus, B. bovis* and *B. bigemina* [12] [13].
2.2. Experimental Design

The trial period was of 24 months (from August 2013 to July 2015) using 50 females submitted to blood collections for quantifications of *B. bovis* and *B. bigemina* infection levels (qPCR), and ticks count once a month. For the calculation of tick infestation, all adult females on the left side of the animal’s body, with size > 4.5 mm in length, were counted. The results obtained after the first year were used to select ten heifers: five presenting the highest levels (H) and five showing the lowest levels (L) of tick infestations and *Babesia* infections levels. At the second experimental year, the previously selected animals were subjected to quantification of *Babesia* infections levels and immune-related transcripts (four samplings, using three months of an interval). All procedures have been approved by EmbrapaPecuáriaSudeste Ethics Committee for Animal Use (CEUA-Embrapa, process number 03/2014), following ethical principles and guidelines of animal experimentation adopted by the Brazilian College of Experimentation. All obtained results of *B. bovis* and *B. bigemina* DNA copies and tick counts from the whole experiment were compared to meteorological data (temperature and rainfall) and had already been previously published [13]. The present study was focused on the comparison of profiles of immune-related transcripts to *Babesia* infections levels.

2.3. Selection of Experimental Animals

The tick counts and DNA copies of *B. bovis* and *B. bigemina* data were transformed into log10 for the normal distribution approximation of the data and then analyzed using the mixed model methods. This model included the fixed effect of collections, the animal random effect, and was used a (co)variance of composite symmetry (CS) to model the animal effect. The predicted values of the animal effects were used to discriminate the five presenting highest (H group) and five presenting lowest (L group) levels of these parasites. The analyses were carried out using the SAS statistical package [14].

2.4. Quantification of Immune-Related Transcripts

Blood samples were collected from the jugular vein, using vacutainer tubes containing EDTA, immediately followed by the transference of 500 µL of collected blood to RNAprotect Animal Blood Tubes (Qiagen, Hilden, Germany) and incubation at room temperature during 2 hr. mRNA extractions from blood samples were performed using RNeasy Protect Animal Blood Kit (Qiagen) according to the manufacturer’s recommendations. The extracted RNA quantification and purity were estimated by 260 nm ultraviolet absorbance and readings at 260/280 nm, respectively. Electrophoresis in agarose gel 1% was used to verify the RNA integrity. Two-step RT-qPCR was used for the relative quantification of gene expression in blood samples. cDNAs were synthesized according to instructions provided with RT2 First Strand Kit (Qiagen) and Oligo (dT) primers (IDT, IA, USA). RT-qPCR assays for relative quantification of iNOS and cytokines RNAm
(IL-12β, IFN-γ, TNFα, IL-10 and iNOS) were carried in CFX96 Touch™ Real-Time PCR Detection Systems (BioRad Laboratories, Hercules, CA, USA) using SsofastEvaGreen® Supermix (BioRad), 100 ng of cDNA and 3.0 pmol of each primer, for a final volume of 13 µL. Amplification included a pre-incubation step at 95°C for 2 min, followed by 45 cycles of 95°C for 5 sec and 60.0°C for 30 sec. After amplification, a melting curve analysis was performed by raising the incubation temperature from 65°C to 95°C in 0.5°C increments with a hold step of 5 sec at each increment. The relative expression of all tested genes (Table 1) was quantified as the fold change relative a calibrator sample, and the gene expression from each sample was standardized using Cq value of the YWAHZ reference gene for the same sample [15]. The stability of the reference gene was tested using three candidates (YWAHZ, GAPDH, and ACTB), and the most stable gene was selected using Reffinder software (http://150.216.56.64/referencegene.php). Efficiencies of RT-qPCR assays were estimated using LingRegPCR software.

The comparisons of the mean relative changes in gene expression and parasite loads between the experimental groups (H and L) were performed using the Mann-Whitney test. These analyses and graphs were performed using GraphPad software (version 8.0), and the level of significance was set at p < 0.05. Correlations between immune response parameters and parasite loads were estimated by Spearman method, using the SAS statistical package [14].

| Gene   | Primers (5' - 3')                                                                 | Accession number | Location (nt) | Product size | Exonboundary | Efficiency | Reference                        |
|--------|----------------------------------------------------------------------------------|------------------|---------------|--------------|--------------|-----------|----------------------------------|
| IFN-γ  | Forward: CAGAGCCAAAATTGTCTCCCTTC  
Reverse: ATCCACCAGAATTTGAATCACG | NM_174086        | 298 - 465      | 167          | 3/4          | 98.02     | Puech et al. (2015)              |
| TNF-α  | Forward: CCAGAGGAAGAGAACGTACC  
Reverse: GGCTCACACCTGGGCTACC | NM_173966        | 367 - 478      | 111          | 3/4          | 98.12     | Puech et al. (2015)              |
| IL-10  | Forward: CTTAAGGTTAAGGTCGAGACG  
Reverse: CTCACCTAGGCCAGCTAGAC | NM_174088        | 219 - 480      | 239          | 2/3          | 98.01     | Puech et al. (2015)              |
| IL-12β | Forward: CAGCAGGCTCTCTGAG  
Reverse: GTGAGGTTTGTATGATCCAGCTG | NM_174356        | 474 - 711      | 237          | 3/4          | 97.99     | Puech et al. (2015)              |
| iNOS   | Forward: CACCTTACTGGAGGAGGATGC  
Reverse: GAACATAGACCTGCTGCTGTTC | DQ676956.1       | 3067 - 3169    | 102          | 5/6          | 99.905    | This study                       |
| GAPDH  | Forward: GCGTGAACCACGAGAAGATTAA  
Reverse: CCCCAGGAGTGAACGATG | NM_001034034.2   | 465 - 583      | 119          | [6]-[7]      | 97.97     | Robinson et al. (2007)           |
| YWAHZ  | Forward: GAAAGGGATTGAGGACCAG  
Reverse: GGCTTCACAAATGTCTGCTCT | NM_174814        | 467 - 650      | 183          | 4/5          | 98        | Puech et al. (2015)              |
| ACTB   | Forward: AGCAAGACGGTGATCGATGAG  
Reverse: ATCCAACCGACTGCTGCTCA | NM_173979        | 1164 - 1402    | 244          | [6]          | 98.01     | Robinson et al. (2007)           |
3. Results

3.1. Selection of Appropriate Reference Gene

YWHAZ was selected as the most stable reference gene from three reference candidate genes tested (GAPDH, ACTB, and YWHAZ) by RefFinder software. All algorithms used by this program pointed YWHAZ as the most stable, except for Genorm algorithm that indicated an association between GAPDH and YWHAZ as the most stable reference gene. On the other hand, ACTB (or BACTIN) was pointed as least stable reference gene by all algorithms used by this program.

3.2. Parasite Levels

*B. bovis* and *B. bigemina* DNA loads were significantly higher (\( p < 0.0001 \) and \( p = 0.006 \), respectively) in H group compared to L group, while no significant difference was observed for tick counts (Figure 1).

3.3. Immune-Related Transcripts

Anti-inflammatory IL-10 cytokine transcripts were found significantly higher in the H group compared to L group (\( p = 0.04 \)). However, no significant differences were observed between these groups regarding TNF\( \alpha \), IFN-\( \gamma \), IL-12\( \beta \), or iNOS-transcripts (Figure 2). Despite the absence of significant differences between experimental groups, IFN-\( \gamma \) transcripts presented a slightly higher median in the H group.

![Figure 1](image1.png)

*Figure 1.* Mean (Log10) of *Babesia bovis* and *B. bigemina* infection and tick infestation measured monthly for 24 months in 10 animals with high (H) or low (L) parasites levels. * = significant difference (\( p < 0.05 \)).
Figure 2. Mean relative expression of iNOS and cytokines (IFNγ, IL-10, IL-12β, and TNFα) in blood samples taken at three-month intervals for one year from 5 animals at low (L) and 5 with high levels (H) of Babesia bovis and Babesia bigemina.

Positive significant correlation coefficients were observed between B. bovis loads and both IL-10 and IFN-γ transcripts ($p = 0.01$ and $\rho = 0.39$; $p = 0.0160$ and $\rho = 0.38$, respectively), while no significant correlation was observed for IL-12β, TNFα or iNOS transcripts, or between B. bigemina loads and all tested immune related-genes.

4. Discussion

Although several studies have been developed regarding immune mechanisms related to resistance against babesiosis, no previous studies are demonstrating differential immune-related transcripts profiles in persistently infected animals showing high or low parasite levels, under natural field challenge. In the present study, although the selection of groups presenting high or low parasite levels intended to include tick counts, B. bovis and B. bigemina infection levels from monitoring period of a year before starting this experiment, no significant difference in tick counts was observed. Weak correlations between Babesia infec-
tion levels and ticks numbers were already demonstrated [13]. We observed significant differences between IL-10 mRNA levels in H and L group in our study. Even, significant positive correlation coefficients between B. bovis loads and both IL-10 and IFN-γ transcripts were also found, while no significant correlations were observed between B. bigemina and immune-related transcripts tested. These findings may be probably derived from the most severe disease induced by B. bovis compared to other babesial parasites [4]. Several authors have found an association between high IL-10 levels and increased susceptibility to B. bovis disease, either by using experimental infection in vivo models or by using in vitro models culturing macrophages or T cell clones [7] [8] [16]. In this context, strong innate immunity to experimental infection with T2Bo strain of B. bovis in calves was associated to the early appearance of IL12β and IFN-γ, while adult cattle succumbed to disease, the expression of these genes was delayed and replaced by IL10 [8]. As revised by Cyktor et al. [17], the majority of intracellular infections, including apicomplexan protozoans are better controlled or cleared faster in the absence of IL10, due to enhanced adaptive immune response, including the production of IFN-γ, reactive oxygen and nitrogen intermediates and pro-inflammatory cytokines. On the other hand, due to its essential immunoregulatory activity associated to downregulation of both Th1 and Th2 responses, IL10 is also necessary to prevent prolonged inflammatory responses that may result in pathologic conditions [4]. In view of IL10 mRNA results here observed, we would expect a counterbalance of pro-inflammatory cytokines, especially IFN-γ, and indirectly, of iNOS transcripts. Though, it was not found, as no significant differences were verified for other immune-related genes tested. It was previously reported that IL10 inhibits IFN-γ protein but not consistently inhibits expression of IFN-γ mRNA, which may be a result of control mechanisms, including accelerated degradation, or altered processing of RNA, which may constitute a reason for our findings [18]. Or the regulatory role of IL10 here observed can be at receptor level instead of transcriptional level, as seen for IL2-receptor p55 in parasite-specific helper T cell clones obtained from cattle chronically infected with B. bovis [16]. IL10 has also been shown to dampen Th1 cell responses to M. tuberculosis infection impairing bacterial clearance, and it was demonstrated that main source of this immunosuppressive cytokine in the early infection are monocytes, while during chronic phase the principal source was replaced by activated effector T cells. Even, mice deficient in T-cell derived IL-10, but not those deficient in monocyte-derived-IL10, presented a significant reduction in bacterial lung loads during chronic infection compared with fully IL-10 competent mice [18]. Polymorphisms in bovine IL-10 or IL-10 receptor gene were already associated with altered susceptibility against mastitis [19] and Mycobacterium avium ssp. paratuberculosis [20].

The present experiment, despite its limitations, showed that IL-10 is differently expressed between animals with higher and lower levels of B. bovis and B. bigemina infections. New experiments will be developed to better clarify the role of this cytokine in resistance to these piroplasms in persistently infected animals.
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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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