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Molecular Characterization of Babesiosis Infected Cattle: Improvement of Diagnosis and Profiling of the Immune Response Genes Expression

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Abstract: The main aim of this study was to improve the detection of *Babesia (B.)* spp. in naturally infected cattle in Egypt. In addition, we analyzed the pattern of expression of selected cytokine genes in response to infection of bovines with *B. bovis* and *B. bigemina*. Infections were detected using both, traditional and novel diagnostic techniques competitive enzyme linked immunosorbent assay (cELISA) and nested polymerase chain reaction (nPCR). Blood samples were collected from 296 healthy Egyptian cattle and examined for babesial infection. Higher prevalence was recorded by cELISA, followed by nPCR, whereas lowest prevalence was recorded by microscopic examination of blood smears. Twenty bovine samples that were found positive for *B.bovis*, twenty samples that were positive for both species (*B.bovis* and *B. bigemina*) using cELISA and nPCR and twenty samples, negative for both species using the same techniques, were selected to carry out a real-time PCR for the quantification of expression of the cytokines genes Interferon gamma (IFN-γ), Interleukin-1 beta (IL-1β) and Transforming growth factor beta 1 (TGF-β1). All of the *Babesia*-positive samples were considered to have sub-clinical infection of babesiosis. The results revealed that infected cattle showed highly significant up-regulation of IL-1β and TGF-β1 genes and down-regulation of IFN-γ gene when compared with non-*Babesia* infected animals. The improvement of babesial infection diagnosis combined with the understanding of the immune complex response will facilitate our understanding of the disease and the development of improved methods for control.

Key words: *Babesia* · Cytokines · cELISA · Nested PCR · RT-PCR

INTRODUCTION

Bovine babesiosis is economically the most important tick-borne disease of cattle caused by protozoan parasites of the genus *Babesia* [1]. *B. bigemina* and *B. bovis* are the main species affecting cattle in Egypt [2]. *Babesia* parasites cause both acute and persistent subclinical disease in cattle. *B. bovis* is responsible for a more acute form of the disease with parasites evading the immune system of the hosts, at least in part, by sequestering in capillaries and antigenic variation mechanisms [3]. The mechanisms involved in *B. bigemina* persistence are less known. Accurate and correct diagnosis of babesial infections plays an important role in monitoring, management and control of infection [4]. Blood smear examination has been considered to be the standard technique for routine diagnosis, particularly in acute cases [5], but not in sub-clinical infections, where the parasitemia is usually much lower [6]. Thus, the occurrence of sub-clinical babesiosis is an important challenge to current diagnostic methods because is difficult to detect. Serological tests, including indirect fluorescent antibody test (IFAT) and ELISA are capable of detecting antibodies in sub-clinical infections. A drawback of these tests is the occurrence of false-positive and false-negative results, involving

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cross-reactive antibodies and/or atypical specific immune responses [7]. Another drawback is that antibodies can be detected even months after recovery of infection though no active infection is prevalent, so these methods cannot help in revealing the exact picture of prevalence of infection at that particular point. A competitive ELISA (cELISA) is an adequate serological tool as it can be easily standardized and less laborious than the IFAT. In addition, it has the potential to display higher specificity than an indirect ELISA [8]. Unlike serological tests, PCR is specific and is able to detect current infections [9]. Another advantage of PCR is that its sensitivity has been reported to be as low as one and three infected erythrocytes. The sensitivity of a PCR assay can be increased several fold by performing a nested PCR [10].

As in other intracellular parasites, a cell-mediated immune response is attributed the most important role in reducing the multiplication of *Babesia* within the host, but also it may cause pathology [11]. An essential role of Th1 cytokine, IFN-γ against *Babesia* during the early or acute stages of disease, has been indicated [12-14]. IFN-γ activates monocytes for increased microbicidal activity, regulates the synthesis of opsonic IgG2 from bovine B-cells and is critical for eliciting host immunity in bovines infected with *Babesia* [15]. Moreover, the pro-inflammatory IL-1β cytokine has been found to inhibit parasite replication *in vitro* [16]. It was reported that Treg cytokine, TGF-β1, seems to have no beneficial clinical effects against acute babesiosis [17]. Cattle that recovered from early or acute *Babesia* infection may sustain sub-clinical infection and become carriers of the parasite, thus potentially threatening other susceptible healthy animals in herds pasturing in endemic areas infested with competent tick vectors. Thus persistent disease helps to perpetuate the cycle of the parasite in nature [7], underscoring the importance for the accurate diagnosis of persistently infected animals. The mechanisms involved in the development of the persistent stage of the disease remain unclear. Yet, it is probable that activation of an immune response that antagonizes the Th1 response is responsible for this change. Although, effects of IFN-γ, IL-1β and TGF-β1 production during acute stage of infection has been addressed in previous studies, there is no information to date on these cytokines levels during the sub-clinical persistent stage of disease in bovines. The aim of the present work was to (1) Study the prevalence of bovine babesiosis in distinct animal populations of Egypt through evaluation the sensitivity, specificity and accuracy of three different diagnostic techniques (blood film examination, cELISA and nPCR) and (2) evaluate the expression of the immune response genes (IFN-γ, TGF-β1 and IL-1β cytokines) in cattle sub-clinically infected with babesiosis using real-time PCR.

MATERIALS AND METHODS

Sampling: The blood samples were collected from 296 apparently healthy Friesian cattle from Sharkeya province and examined for diagnosis of babesiosis. Each sample was divided into two parts; one of them was anticoagulated with EDTA for DNA and RNA extraction for PCR assays and the second without anticoagulant for serum separation for cELISA assay.

Analysis of Samples:

Blood Film Examination: Blood smears from all animals were prepared and stained with Giemsa following the standard method [18]. The stained smears were examined by light microscopy (Nikon Eclipse, E400, Japan) at x100.

Competitive ELISA: The antibody Test Kit, cELISA used was gently provided by VMRD (Pullman, WA). It is based on recognition of a B-cell epitope present in the Rhoptry Associated Protein-1 (RAP-1) of either *B. bovis* or *B. bigemina* that is defined by monoclonal antibodies (mAb) [19]. The testing was performed according to the manufacturer's instructions. Optical density (O.D.) values were determined using EL_X 800 Universal microplate reader Bio-TEK instruments, INC, USA. The results were expressed as a value of the percent inhibition (%I) according to the following formula:

(%I): %I = 100 - [(Sample O.D. × 100) / (Mean negative control O.D.)].

Samples were classified as positive if the %I value was above 40% and negative if the %I value was less than 40%. The manufacturer of the kit established these cut-off values [20, 21].

PCR and Nested PCR on Blood Samples: 10 µl of total blood from all tested animals and controls was spotted on Whatman FTA® Elute cards (Cat. No. WB120410) and the materials were processed for DNA elution following the manufacturer's instructions. 2-3 µl of eluted solution were used in 25 µl of PCR mixture.

PCR was performed in a final volume of 25µl containing 12.5µl JumpStart RED Taq Ready Mix PCR reaction mix (Sigma-Aldrich), 1µl of each primer (10 pmol, Metabion, International AG) and 8.5µl sterile water. Two microliter of template DNA was used for the primary PCR. The nested PCR utilized 0.5 µl of primary PCR product as template.

The PCR amplifications were performed in a thermocycler (PTC-100, BioRad, USA). The conditions for *B. bovis* primary PCR were 95°C for 3 min followed by 25 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min and extension at 72°C for 1 min then a final extension step at 72°C for 5 min. The conditions for the *B. bovis* nPCR were identical to the primary PCR except the number of cycles was 35 cycles.

The conditions for *B. bigemina* primary PCR were 95°C for 3 min followed by 25 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 61.2°C for 30 sec and extension at 72°C for 30 sec then a final extension step at 72°C for 5 min. The conditions for the *B. bigemina* nPCR were 95°C for 3 min followed by 25 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 63.1°C for 30 sec and extension at 72°C for 30 sec then a final extension step at 72°C for 5 min. Positive control and negative control (with no DNA template) were always included for PCR amplification. Oligonucleotide primer sequences and expected amplicon size used in PCR assay were showed in Table (1). Amplification products were electrophoresed on 1.5% agarose gel stained with ethidium bromide using 50 bp and 100 bp DNA ladders as size marker (Fermentas, Germany). They were visualized under UV trans-illuminator (Bachofer, D7410) and photographed using gel Documentation system (BioDocAnalyze-Biometra Analytic GmbH).

Quantification of Cytokines Expression

RNA Extraction: Total RNA was extracted from buffy coat from 296 apparently clinically healthy cattle using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following manufacturer's instructions. An aliquot of total RNA diluted in RNase free water was set aside to estimate RNA quantity and integrity and the remaining sample was stored at -80°C until gene expression analysis. The concentration and purity of the RNA samples was determined using a Nano-Drop® (ND-1000 Spectro-photometer, Nano-Drop Technologies Inc, Delaware, USA). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis and all samples maintained a 28S/18S rRNA ratio of 1.8 or greater.

cDNA Synthesis: cDNA synthesis was carried out on RNA extracted from twenty *Babesia*-positive samples in both nPCR and cELISA for *B.bovis* and positive twenty samples in both nPCR and cELISA for mixed infection with *B.bovis* and *B.bigemina* as well as negative twenty samples. The RNA of these samples was treated with DNase (Fermentas, EU) to remove any possible DNA contamination according to the manufacturer's instructions. The DNase treated RNA was reverse transcribed into First Strand cDNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, EU) according to the manufacturer's instructions.

Real-time PCR (RT-PCR): Real-time PCR was performed for quantification of cytokines genes, IFN-γ, IL-1β and TGF-β1. Real-time RT-PCR was performed using the Cepheid SmartCycler® II system (Sunnyvale, CA, USA). Intron-spanning gene-specific primers that were short enough to ensure optimum amplification were chosen

Table 1: Oligonucleotide primer sequences, gene name and expected amplicon size used in PCR assay.

Parasite	Primer Name	Sequence	Product Size	Gene name	Reference
Babesia	BoFP	5´-CACGAGGAAGGAACTACCGATGTTGA-3´	823 bp	*B. bovis*	Suarez *et al*.
Bovis	BoRP	5´-CCAAGGAGCTTCAACGTACGAGGTCA-3´		RRA gene	(2011)
	BoFN	5´-TCAACAAGGTACTCTATATGGCTACC-3´	387 bp		
	BoRN	5´-CTACCGAGCAGAACCTTCTTCACCAT-3´			
Babesia	BiFP	5´- ATGATTCACTACGCTTGCCTC-3´	600 bp	*B. bigemina*	Suarez *et al*.
Bigemina	BiRP	5´- GTCTTGTAGTATATGGCGGTCATGTAG-3		RAP-1 gene	(2003)
	BiFN	5´- TCTCGAAGACAGCGAACAGA-3´	237 bp		
	BiRN	5´- GTGAAGCTGGTAGGGGTCAG-3´			

Bo: *bovis*, Bi: *bigemina*, F: forward, R: reverse, P: primary, N: nested, RRA: Rhoptry associated protein related antigen, RAP-1: Rhoptry associated protein-1

Table 2: Oligonucleotide primer sequences, amplicon size and the primer references used in real-time PCR assay.

Gene	Primer sequence	Amplicon size(bp)	Reference
IFN-gamma	F : 5-AGC CAA ATT GTC TCC TTC TAC TTC-3		
	R :5- CTG ACT TCT CTT CCG CTT TCT G-3	261 bp	Karcher *et al*. (2008)
TGF beta1	F : 5-GGA CCT GGG CTG GAA GTG-3		
	R :5- CTG CTC CAC CTT GGG CTT-3	222 bp	Plath *et al*. (1997)
IL-1 beta	F: 5-ATC TTC GAA ACG TCC TCC GAC-3		
	R :5-CCT CT CCT TGC ACA AAG CTCA-3	187 bp	Buza *et al*. (2003)
GADPH	F: 5-CCT GGA GAA ACC TGC CAA GT-3		
	R :5- GCC AAA TTC ATT GTC GTA CCA-3	214 bp	Buza *et al*. (2003)

from published references (Table 2). The 25µl reaction mixture consisted of 12.5 µl SYBR Green PCR master mix (Applied Biosystems, USA), 0.5 µl of each primer (10 pmol), 1µl cDNA (400ng) and 10.5 µl RNase free water.

For each gene of interest, negative and positive controls were included. For glyceraldehyde-3-phosphate dehydrogenase (GADPH), IFN-γ and IL-1β genes, forty cycles of amplification with denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 60°C for 30 sec, were performed after an initial incubation at 95°C for 5 min, while for TGF-β, forty cycles of amplification with denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 60°C for 1 min, were performed after an initial incubation at 95°C for 5 min. For each sample a melting curve was generated after completion of amplification and analyzed in comparison to the positive and negative controls. Mean cycle threshold (Ct) values of duplicate samples were used for analysis.

Data Analysis: The chi-square test was used to evaluate significant differences ($P<0.05$) of infection rate between the three diagnostic methods. Data from the real-time PCR were analyzed using the $2^{-\Delta\Delta Ct}$ method [24]. In this study, GAPDH served as the housekeeping gene to calculate the ΔCt. Statistical significance was evaluated using the Student's *t*-test (Microsoft Excel). A P value < 0.05 was considered statistically significant.

RESULTS

Diagnosis of *Babesia* spp using microscopic examination, cELISA and nPCR: A total of 296 blood samples was randomly collected from cattle apparently healthy and tested for the presence of *B. bigemina* and *B. bovis* using microscopic examination, cELISA and nPCR. The results of the microscopic observation for babesiosis revealed that only 33 animals were infected with *Babesia spp.* with overall prevalence rate of 12%.

The cELISA analysis showed that out of 296 serum samples tested, 130 (44%) represented a positive result to specific antibodies of babesiosis, with 64 samples (21.6%) positive for *B. bigemina*, 32 samples (10.8%) positive for *B. bovis* and 34 samples (11.6%) positive for both species (mixed infection).

The molecular analysis revealed that, in the primary PCR no bands were visible, possibly due to the low levels of parasitemia. However, in nPCR, 117 (39.5%) samples were positive for babesiosis in which 73 (24.7%) were positive for *B. bigemina* and 44 (14.8%) samples, were positive for *B. bovis*. The expected 387- and 237-bp fragments for *B. bovis* and *B. bigemina,* respectively, were detected using agarose gel electrophoresis (Figure 1, A and B).

Therefore, a higher prevalence of babesiosis was recorded by using cELISA, followed by nPCR, whereas, the lowest prevalence was recorded by microscopic examination of blood smears.

Using the Chi-square test, it has been found that the differences between the three methods were statistically significant as there was pair wise variation between infected animals.

Gene Expression Analysis for Ifn-γ, Il-1β and Tgf-β1 Genes: Twenty samples, positive for *B.bovis* in the nPCR and cELISA*;* twenty samples, positive for both species (Mixed infection) in the nPCR and cELISA and twenty samples, negative for both species in the three techniques*,* were selected to carry out a real-time PCR for the quantification of cytokines genes, IFN-γ, IL-1β and TGF-β1. All the positive samples were considered to have sub-clinical infection of babesiosis. All genes presented a single peak in the PCR melting curve, which indicates absence of primer-dimer formation during the reaction and specificity of the amplification (data not shown). Fig (2) showed the N-fold change of cytokines genes expression in the population of cattle sub-clinically infected with *Babesia* cattle compared to the population of non-infected (Control) cattle.

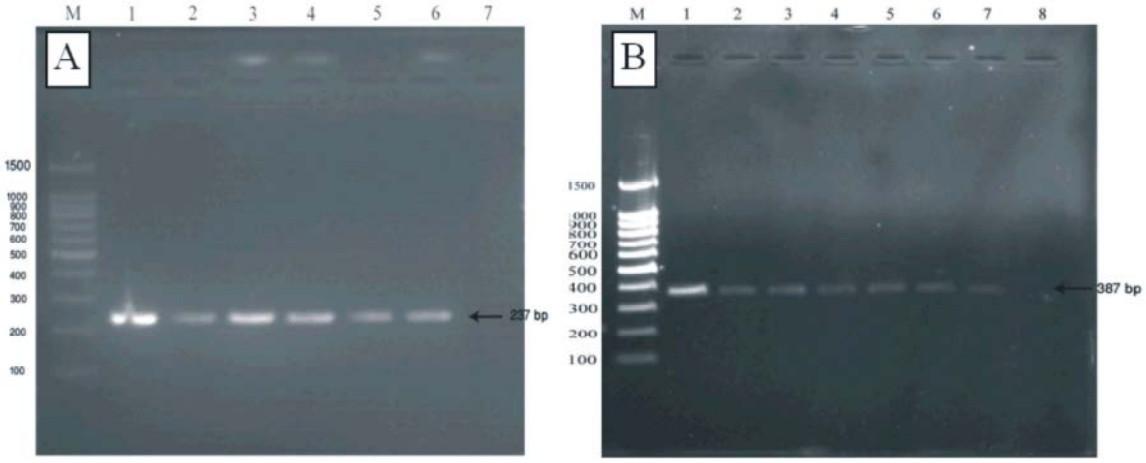

Fig. 1: Ethidium bromide-stained agarose gel of PCR amplified fragments using primers which are specific for: *B.bigemina* (A) and *B. bovis* (B). 100-bp DNA ladder run in lane M; lane 1: represents positive control; lanes 2-6: represent positive blood samples; lane 7: represents negative control (double distilled water); the *arrow* showed the PCR amplified fragment.

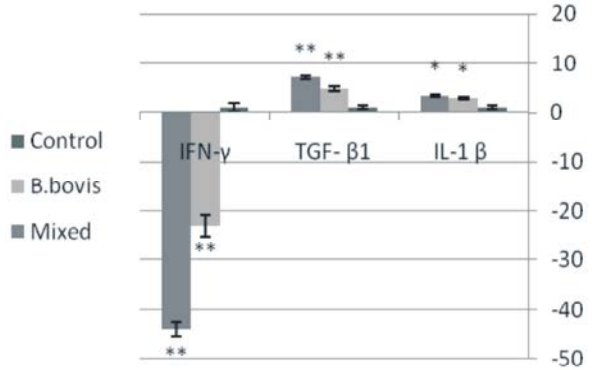

Fig. 2: N-fold change of cytokines genes expression in babesiosis sub-clinically infected cattle compared to healthy (control) cattle. Bars represent standard error. * =P<0.05; ** = P<0.01

Interestingly, the population of animals with sub-clinical babesiosis shows a pattern of significantly decreased levels of expression of the IFN-γ cytokine gene when compared to the non-*Babesia* infected animals. Thus, taking together, the IFN-γ mRNA levels were significantly down-regulated by 44 fold (P<0.01) in *B.bovis* infected animals and significantly down-regulated by 22.9 fold (P<0.01) in mixed infection compared to control non-infected animals.

In contrast, compared to the non-*Babesia* infected animals, the IL-1β mRNA levels were significantly up-regulated by 2.9 fold (P<0.05) in *B.bovis* infected animals and significantly up-regulated by 3.4 fold (P<0.05) in animals infected with both *Babesia* species.

In addition, the animals with sub-clinical babesiosis also have a significant increase in TGF- β1cytokine gene expression when compared to the non-*Babesia* infected animals. Thus, overall, TGF- β1mRNA levels were significantly up-regulated by 4.8 fold (P<0.01) in the population of *B.bovis* infected animals and significantly up-regulated by 7.1 fold (P<0.01) in the population with mixed *B. bovis* and *B. bigemina* infection.

DISCUSSION

Babesia bovis and *Babesia bigemina* are the main causative agents of bovine babesiosis in Egypt [28]. *B. bovis* is the most virulent species, but in most areas of endemicity, *B. bigemina* is more prevalent. Both species can cause clinical disease and sub-clinical infections [21]. In Egypt, large numbers of cattle are infected with sub-clinical babesiosis, but the actual prevalence of the disease remains unknown [29, 30]. This sub-clinical state is challenge to current diagnostic methods and is difficult to detect because of the low number of parasites in circulation. However, diagnosis of carrier animals in herd is important for preventing outbreaks by transmission through vector ticks to healthy animals and for obtaining epidemiological data of disease [31].

During the present study, a total of 296 blood samples was randomly collected from cattle apparently healthy from the Sharkeya province in Egypt and tested for the presence of *B. bigemina* and *B. bovis* using microscopic examination, cELISA and nPCR. The results of the microscopic examination for babesiosis revealed

that only 33 animals were infected with *Babesia* spp. with overall prevalence rate of 12%. Similar results were obtained in Egypt by many authors namely Adham *et al.* [28], El-Ghaysh [32], Adel [33] and El-Fayomi *et al.* [34], who reported *Babesia* spp. infection rate of 13% in Giza Governorate, 12.5% in Cairo, 11.1% in Gharbia Governorate and 13% in Port Said Governorate, respectively. Also, Abd-El-Gawad [35] and Mazyad and Khalaf [36] reported infection rate of 9.9% and 8.1% in Beni-Suef and North Sinai Governorates, respectively. Traditionally, microscopic examination has been considered the "Gold standard" for detecting *Babesia* organisms in the blood of infected animals, particularly in acute cases, but not in carriers, where the parasitemia is low [6].

For our knowledge, this is the first study in Egypt using recombinant protein based cELISA for detecting the prevalence of *Babesia* spp in cattle. These tests are based on the ability of serum antibody to inhibit a monoclonal antibody (MAb) directed against *Babesia* species-specific epitopes within the C terminus of the rhoptry-associated protein 1a (RAP-1a) either of *B. bovis* or *B. bigemina*. Goff *et al.*, [21] reported that the specificity of cELISA was 98.3% and sensitivity was 94.7%. Our results indicated that 44% of examined serum samples from the Sharkeya province were sero-positive, suggesting a high rate of bovine *Babesia* infection occurring in Egypt. Specific antibodies of *B. bovis* and *B. bigemina* were detected in 11% and 21% respectively. However, 12% of the samples analyzed in this study showed mixed infection of both species. Ibrahim *et al.* [2] reported that the prevalence of *B. bigemina* and *B. bovis* was 10.60% and 9.27% using indirect ELISA, respectively, in Beheira, Egypt.

Molecular identification based on nested PCR amplification technique used as the most specific and accurate method in diagnosis of babesiosis especially in subclinical phase [37]. Our results of nPCR revealed that 117 (39.5%) samples were positive for babesiosis in which 55 (18.6%) were positive for *B. bigemina*, 32 (10.8%) were positive for *B. bovis* while 30 (10.1%) were positive for both species. In Egypt, El-Fayomi *et al.* [34] and Rania [38] reported infection rate of 23% and 25.3%, respectively. In Pakistan and Philippines, Chaudhry *et al.* [31] and Longzheng *et al.* [39] used nPCR for diagnosis of *Babesia* spp. in cattle and found infection rate of 29% and 18.8%, respectively. On the other hand, Silva *et al.* [40] in Portugal found infection rate of 78.5%. Fluctuation in the prevalence rates might be due to the variance in animal locality and the season of study. Several studies reported that humidity plays an important role in tick population density [41] and an increase in temperature may allow vectors to migrate into new areas or to allow a significant development of parasites [34, 42].

The present results showed high sensitivity of nPCR technique (39.5%) in detection of *Babesia* spp. as compared to microscopic examination (12%). In agreement with our results, Mahmmod [30], Shams *et al.* [37] and Baldani [43] indicated that nPCR assay is more sensitive technique than microscopic examination for detection of *Babesia* spp. in apparently healthy cattle which suggests its use as a routine technique for diagnosis of sub-clinical bovine babesiosis. It is noted that, cELISA assay detected a higher number of infected animals (44%) than the nPCR assay (39.5%); this difference underlines the fact that they detect different entities (Parasite-specific antibodies / parasite DNA) which have opposite time response after infection. Furthermore, persistently infected animals may contain a very low number of parasites in circulation, thus making their detection by PCR more difficult [44, 45]. Similar results obtained by many authors, Ibrahim *et al.* [29] in Egypt, Silva *et al.* [40] in Portugal, Terkawi *et al.* [46] in Thailand and Dominguez *et al.* [8] in Argentina, who recorded a higher babesial infection rate using ELISA assay compared to that using nPCR.

B. bigemina infection seemed to be more frequent in accordance with previous works noting that in Africa *B. bigemina* is more prevalent than *B. bovis* due to the tick distribution [47]. Similar finding was observed by Ibrahim *et al.* [29], reported that *B. bigemina* is more prevalent than *B. bovis* in Egypt. Also, Adham *et al.* [28] reported that prevalence of *B. bigemina* is higher than *B. bovis* in *Rhipicephalus annulatus* in Egypt. In Mediterranean countries like Egypt, *Rhipicephalus (Boophilus) annulatus* is known to be the most prevalent tick and the main vector transmitting *B. bigemina* and *B. bovis* [48, 49].

A general lack of understanding of the mechanism by which immunity to *Babesia* infection occurs contributes to the slow development of novel therapeutics [10, 50]. The immune response to the infecting parasites is complex and involves both the humoral and cellular branches of immune systems [51]. Because cytokines constitute an important link between humoral and cellular immune response, their role in the host response against parasites may be very important. To the best of our knowledge, this is the first study to address the gene expression of IFN-γ, TGF-β1 and IL-1β cytokines in *Babesia* naturally infected cattle through sub-clinical phase. In the current study, *Babesia* sub-clinically infected cattle tended to have significantly

IFN-γ down-regulation (P<0.01) compared to the non-*Babesia* infected cattle. Previous studies on murine and bovine babesiosis reported a high expression level of IFN-γ in the primary infection and through the acute phase of the disease [12-14, 52]. On the other hand, we found that TGF-β1 mRNA levels were significantly higher (P<0.05) than those in non-*Babesia* infected animals. Overall, these data is consistent with an inhibition of the Th1 pattern (IFN-γ) through an enhancement of Treg pattern (TGF-β1). The previous results are consistent with Aung *et al.* [53], Sasindran and Torelles [54] and Maia and Campino [55], who reported that TGF-β1 mRNA level tented to elevate in sub-clinically bacterial and intracellular parasitic infections. Also, we found that IL-1β mRNA levels were significantly higher (P<0.05) than those in healthy animals. Shoda *et al.* [16] described IL-1β role in *B.bovis* infection. They reported that *B. bovis*-stimulated macrophages expressed enhanced levels of interleukin-1beta to inhibit parasite replication *in vitro*. A positive regulation was found between IL-1β and TGF-β1 [56- 59] which suggests that IL-1β may be up-regulated in response to TGF-β1up-regulation. De Boer *et al.* [60] and Eigenbrod *et al.* [61] demonstrated decrease of IL-1β production after IFNγ stimulation through different mechanisms. This information suggests that the down-regulation of IFN-g gene expression noted in sub-clinically infected animals may be not solely a result of a shift to Treg type response (TGF-β1 gene). Rather, the increased gene expression of IL-1β may be involved.

CONCLUSION

The combination of cELISA and nPCR is considered to offer the greatest sensitivity for babesial diagnosis especially through the sub-clinical phase. Containment of babesial infection during the sub-clinical phase may be achieved through the up-regulation of TGF-β1, a Treg cytokine and IL-1β that accompanied by the down-regulation of IFNγ, a Th1 cytokine.

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