Identification of interchangeable cross-species function of elongation factor-1 alpha promoters in Babesia bigemina and Babesia bovis

Marta G. Silva 1*, Donald P. Knowles 1,2 and Carlos E. Suarez 1,2

Abstract

**Background:** Tick-borne Babesia bigemina is responsible for acute and potentially lethal hemolytic disease in cattle. The development of genetic manipulation tools necessary to the better understanding of parasite biology is currently limited by the lack of a complete parasite genome and experimental tools such as transfection. Effective promoters, required to regulate expression of transgenes, such as the elongation factor-1 alpha (ef-1α), have been identified in other apicomplexans such as Babesia bovis and Plasmodium falciparum.

**Methods:** The B. bigemina ef-1a locus was defined by searching a partial genome library of B. bigemina (Sanger Institute). Presence of an intron in the 5′ untranslated region was determined by 5′ Rapid Amplification of cDNA Ends (RACE) analysis. Promoter activity was determined by measurement of luciferase expression at several time points after electroporation, efficiency of transfections and normalization of data was determined by quantitative PCR and by the percentage of parasitized erythrocytes.

**Results:** The ef-1a locus contains two identical head to head ef-1α genes separated by a 1.425 kb intergenic (IG) region. Significant sequence divergence in the regions upstream of the inverted repeats on each side of the B. bigemina IG region suggest independent regulation mechanisms for controlling expression of each of the two ef-1α genes. Plasmid constructs containing the 5′ and 3′ halves of the IG regions controlling the expression of the luciferase gene containing a 3′ region of a B. bigemina rap-1a gene, were generated for the testing of luciferase activity in transiently transfected parasites. Both halves of the ef-1α IG region tested showed the ability to promote high level production of luciferase. Moreover, both B. bigemina ef-1a promoters are also active in transiently transfected B. bovis and conversely, a B. bovis ef-1a promoter is active in transiently transfected B. bigemina.

**Conclusions:** Collectively these data demonstrate the existence of two distinct promoters with homologous and heterologous promoter function in B. bigemina and B. bovis which is described for the first time in Babesia species. This study is of significance for development of interspecies stable transfection systems for B. bigemina and for B. bovis.

**Keywords:** Babesia bovis, Babesia bigemina, Transfection, Elongation factor-1α
Background

Tick and tick-borne diseases (TBDs) have vast impact on food production and public health. Bovine babesiosis is recognized among the most important TBD in terms of economic losses worldwide [1]. Bovine babesiosis can be in part controlled using live attenuated vaccines, but these vaccines are challenging to produce and distribute, have safety risks and a limited shelf life [2]. The existence of numerous research gaps concerning host-parasite relationships limit options for the development of improved methods for control. Novel research tools are needed in order to close such knowledge gaps and support development of new methods to control Babesia parasites and its vectors [3]. Babesia bovis and B. bigemina are the main parasites responsible for bovine babesiosis in terms of global parasite distribution, while Babesia divergens is mainly prevalent in the European continent [1].

In general B. bovis is regarded as the most virulent Babesia parasite, whereas, B. bigemina causes acute hemolytic disease [4]. Although the B. bovis genome and transfection systems were developed several years ago [5, 6], the genome for B. bigemina remains incomplete and unassembled (http://www.sanger.ac.uk/resources/downloads/protozoa/babesia-bigemina.html), and gene editing and transfection systems for B. bigemina remain unavailable. Attenuated transected Babesia parasites are ideal candidates for delivery of anti-tick or other anti-TBD agents’ vaccine antigens [7]. Considering that B. bigemina has a wider range of tick vectors than B. bovis, including Rhipicephalus (Boophilus) microplus [8], B. annulatus [9] and B. decoloratus [10, 11] this parasite should also be considered as an efficient candidate for the development of dual parasite-vector vaccine approaches.

Gene editing and transfection systems, in addition to a complete genome, are required in order to advance our understanding of the molecular biology of B. bigemina parasites and for improved vaccine development. A current limitation for the development of such gene analysis systems is the lack of defined B. bigemina promoters and a method to introduce foreign DNA into B. bigemina. The promoters controlling the expression of elongation factor 1-alpha (ef-1α) genes in apicomplexan parasites are generally regarded as strong constitutive promoters [12, 13]. In B. bovis the ef-1α includes an arrangement of two identical head to head genes separated by a 1.4 kb region containing two apparently distinct promoters [12, 13]. Therefore a rational approach for the identification of B. bigemina promoters consists of searching for the presence of a similar structural and functional arrangement for the ef-1α locus in this parasite.

Given the current unavailability of a genetic system to better characterize the molecular basis for B. bigemina virulence and to facilitate vaccine development, we proposed to define promoters and a method to introduce foreign DNA into the parasite. In this study we describe the ef-1α locus of B. bigemina, and tested the activity of ef-1α promoters. The data generated in this study may serve as the foundation for the future development of urgently needed gene editing and stable transfection systems for B. bigemina.

Methods

Parasites

The B. bigemina Puerto Rico strain [14] and B. bovis Texas S74T3Bo strain [15] were grown in long-term microaerophilous stationary-phase (MASP) culture using previously described methods [16, 17]. Parasites were obtained from 4 12.5 cm²-flask expansions of B. bigemina cultures containing approximately 25 % infected red blood cells (iRBC) as determined by microscopic counting of Diff-Quick-stained (Dade Behring, Deerfield, USA) slides. Infected red blood cells from the expansion were centrifuged for 10 min at 400×g. The cell pellet was suspended in 4 ml of Cytomix (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl₂, pH 7.6) and centrifuged again for 10 min at 400×g. The supernatant was removed and the tube of packed red blood cells was placed on ice until needed (Electroporation sub-section).

Parasite DNA extraction

Genomic DNA (gDNA) was extracted from cultured B. bigemina Puerto Rico strain and cultured B. bovis Texas strain using the Qiagen Blood core kit (Valencia, USA) following the manufacturer’s instructions. The same kit was used to extract plasmid DNA (pDNA).

Identification and sequencing of the Babesia bigemina ef-1α locus of the Puerto Rico strain

BLAST searches of the unfinished B. bigemina genome at the Sanger website (http://www.sanger.ac.uk/resources/downloads/protozoa/babesia-bigemina.html) were performed using the B. bovis ef-1α orf as a query (GenBank accession number: ALH43162.1). The full sequence of the ef-1α gene A in the B. bigemina Puerto Rico strain was determined by PCR analysis using the ef-1α orf reverse primer (Ef1α-Rev): 5’-CTT CTT GGA GGC CTT CTG GGC TG-3’ in conjunction with a primer present in the IG region, Ig-A-forward (Ig-A-Fwd): 5’-ATT AGG AAG CTT TAC AGA GGA CAT ACT TTA CTT G-3' containing a HindIII restriction site at 5’ end. Primers Ig-A-Fwd and Ef1α-Rev and Ig-B-forward (Ig-B-Fwd): 5’-ATT AGG AAG CTT CAA GTA AAG TAT GTC CTC TG-3’ containing a HindIII restriction site at 5’ end and Ef1α-Rev were used to amplify both halves of the full ef-1α locus (Fig. 1). Both 2.075 and 2.063 kb amplicons were cloned into TOP-TA pCR2.1

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**Primers used in this study:**

- Ef1α-Rev: 5′-ATT AGG AAG CTT TAC AGA GGA CAT ACT TTA CTT G-3′
- Ig-A-Fwd: 5′-ATT AGG AAG CTT CAA GTA AAG TAT GTC CTC TG-3′
- Ig-B-Fwd: 5′-ATT AGG AAG CTT CAA GTA AAG TAT GTC CTC TG-3′

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cloning vector (Life technologies, Carlsbad, USA) and fully sequenced.

Transfection plasmid constructs

*Babesia bigemina* gDNA was amplified using primers to amplify the Ig-A and Ig-B IG regions. For the Ig-A region a set of primers Ig-A-Fwd and IG-FS-reverse: 5′-CTTCTTGGAGGCCTTGCTGTCG-3′, each containing a *Hind*III restriction site at the 5′ end, were used to amplify a 709 bp fragment. For the Ig-B region the set of primers Ig-B-Fwd and IG-FS reverse (sequence described above), each containing a *Hind*III restriction site at the 5′ end, were used to PCR amplify a 716 bp fragment. PCR products from both promoters were cloned into the TOP-TA pCR2.1 cloning vector (Life technologies) for sequence confirmation. Both Ig-A and Ig-B regions were then digested from the pCR2.1 plasmids with *Hind*III restriction enzyme (Promega, Madison, USA) and ligated into the linearized *Hind*III restriction site at the 5′ end of the *B. bigemina* rap-1 locus. All plasmids were sequenced to confirm that the promoters were in the correct orientation. A small 245 bp region of the 3′ region of *B. bigemina rap-1a* gene was amplified from gDNA of *B. bigemina* using the set of primers 3′ rap-a forward: 5′-GCG TCT CTG CAG TAA CAA TTT AGC TGT AC-3′ and 3′ rap-a-reverse: 5′-CCG TAA CTG CAG ACA CGC TAT CTA CGG TGG TGG C-3′, each containing a *Pst*I restriction site at the 5′ end. The *B. bovis* 3′ rap-1 region of the “4-35-ef-luc” plasmid was removed by digesting with *Pst*I restriction enzyme and replaced by ligation with the 245 base pair rap-1a 3′ region from the rap-1a gene of *B. bigemina* utilizing the same *Pst*I restriction site.

**RACE analysis: Analysis of *B. bigemina* ef-1a gene A transcripts**

The *B. bigemina ef-1a* cDNA was specifically amplified using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, USA) protocol, the Advantage 2 PCR enzyme system and universal primer mix (UPM): 5′-CTA CTA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT-3′. Primers race-1-ef-1a: 5′-CGT TCT TCT AGT CAA TGG CTA CGA CCA TGG GAG ATT-3′; race-2-ef-1a: 5′-GTA CCG TTG TCC ATA CGG CTG GTG ATC-3′; and race-3-ef-1a: 5′-CCG TGG CAA CAT CGA TAA CTA CTG GTG ATC-3′ were used for specific amplification of *B. bigemina ef-1a* gene A transcripts and amplified 5′ RACE products were cloned in vector 2.1-TOPO and sequenced.
Electroporation
All electroporations were performed in a Gene Pulser II apparatus (BioRad, Richmod, USA) using 0.2-cm cuvettes. Twenty μg of plasmid was dried down in a Speedvac (Savant, Pittsburgh, USA) and suspended in 25 μl of Cytomix. Then, 37.5 μl of packed RBC were suspended in an equal volume of Cytomix, mixed with the plasmid briefly and transferred to a 0.2 cm cuvette follow electroporation. The conditions for electroporation were: 1.2 kV, 200 Ω and 25 μF. After electroporation, the cells were transferred to the well of a 48-well plate containing 5 % normal RBCs and HL-1 media with 40 % bovine serum to a final volume of 465 μl. The plate was then placed in the incubator until the luciferase assay was carried out. After electroporation, merozoites were cultured in vitro as described above.

Luciferase assays
Luciferase analysis was performed as previously described in Suarez et al. [18]. Briefly, luciferase assays were performed using Promega’s LAR II detection reagent at room temperature and a Turner Designs TD-20/20 Tube Luminometer for a 10 s integration interval.

Quantitative real-time PCR
Real-time PCR analysis of the treatment groups. Copy numbers of luciferase, B. bigemina ccp-3 and B. bovis msa-1 genes were calculated based on a standard curve as previously described [19]. Statistical significance was analyzed using a two-sample t-test for differences among the treatment groups.

Results and discussion
Features and full sequence of the ef-1a locus in B. bigemina
The search for the B. bigemina Bond strain ef-1a locus identified a single contig (contig 4117.1) containing a truncated ef-1a orf followed by a 1.4 kb intergenic (IG) region and a full size ef-1a orf (Fig. 1). These data suggest that the B. bigemina genome contains an ef-1a locus which is structurally similar to the locus found in B. bovis and Plasmodium [12]. A full and identical ef-1a gene copy A was found to be located in the 5′ region upstream of the incomplete sequence reported in the Sanger database in the Puerto Rico B. bigemina strain. Further sequence analysis of the ef-1a locus of the Puerto Rico B. bigemina strain showed a putative glutamyl tRNA synthase gene located immediately upstream to the ef-1a-B gene (GenBank accession number XM_012911268.1). Synteny among these two genes is also conserved in the B. bovis and Plasmodium genomes [12]. Based on this information, we also obtained the full sequence of the ef-1a gene A and confirmed the full B. bigemina ef-1a locus structure by performing PCR amplifications on the Puerto Rico strain of B. bigemina (Fig. 1).

Full sequencing of the PCR products (Lane 1, Fig. 1) revealed an ef-1a orf A which is identical to the ef-1a B gene (Lane 2, Fig. 1), and provided novel sequence data for the IG region and ef-1a gene B, with a few base discrepancies when compared to data of the unfinished genome (GenBank accession number KT439182). Overall, analysis of the PCR products confirmed that B. bigemina contains an ef-1a locus which is identical in
structure to the *B. bovis* locus, consisting of two identical 1.347 kb ef-1α orfs which are separated by a 1.425 kb IG region as represented in the top panel of Fig. 1. Furthermore, BLAST searches performed with the full size ef-1α locus deduced from the Puerto Rico strain (GenBank accession number KT439182) shows 99% identity with a 4.119 kb region located in the assembled Chromosome 1 of *B. bigemina* Bond strain (bp 1849423-1853541) (GenBank accession number LK391707).

While the sequences of the ef-1α orfs from *B. bovis* and *B. bigemina* are highly similar (88% identity), the sequence of the *B. bigemina* IG region is unrelated (no significant homology detected) to the *B. bovis* IG region. Yet, the *B. bigemina* and the *B. bovis* ef-1α IG regions have similar structural features, including the presence of inverted repeats (IR) (Figs. 1 and 2a). The promoter activity of two fragments termed Ig-A and Ig-B, derived from each half of the ef-1α IG region of *B. bigemina* was tested using luciferase assays. The
transient transfection plasmid contained either the fragment Ig-A representing 712 bp upstream of the ef-1α orf A, or the fragment Ig-B representing 713 bp upstream of the start of the ef-1α orf B (Fig. 2a). The plasmid vectors using in the transfections are shown in Fig. 3a and b. Luciferase expression reached a peak at 8 h after electroporation, regardless of the IG region used as promoter (Fig. 3a). The results comparing expression of luciferase at 8 h post-electroporation for each construct are shown in detail in Fig. 3b (\( F(5,12) = 20.63, P = 0.000 \)). Comparative growth curves and statistical analysis (Fig. 3a and Additional file 1: Figure S1a) shows that *B. bigemina* viability at 8 h post-electroporation was equally affected among all transfected plasmids regardless of the plasmid used for electroporation (\( F(6,14) = 0.75, P = 0.617 \)). In addition qPCR demonstrated similar efficiencies of transient transfection for all plasmid/parasite combination used in the study. The qPCR was performed using the *B. bovis* 3′ termination *msa*-Sequence, which is unique to the transfection plasmid, *ccp3*, a single gene copy of the

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![Fig. 3](image-url) **Fig. 3** a Homologous function of the *B. bigemina* IG promoters. Functional promoter analysis of the *B. bigemina* IG region in *B. bigemina* transfected parasites. The Y axis represents the percentage of parasitized erythrocytes (PPE), and the Z axis represents Relative Luciferase Units (RLU). The X axis represents the different plasmid constructs (Ig-A, Ig-A Rev, Ig-B, Ig-B Rev, 4-35-ef-luc, Pr-, pBluescript) transfected and the control (4-35-ef-luc not transfected) at the time of the measurements performed at different hours post-electroporation. The black bars represent the PPE ± standard deviation (SD). The grey line represents relative luciferase units (RLU). b Schematic representation of the plasmid constructs used in the transient transfections and the RLU obtained eight hours after electroporation. Asterisks represent statistically significant differences detected using the t-test \((P < 0.01)\)

![Fig. 4](image-url) **Fig. 4** Efficiency of transient electroporation of *B. bigemina*. The number of plasmid DNA copies per genome was calculated from real time PCR analysis at eight hours after electroporation. The Y axis represents the ratio of plasmid/genome copies (Luc/genome copies) and the X axis represents the different plasmid constructs used in the electroporation. No significant differences were detected among the different plasmids electroporated into *B. bigemina*. 

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*B. bigemina* genome and the *luc* gene [20] (Fig. 4). No significant differences in the number of plasmids per genome were found among the distinctly transfected parasites indicating that the ratios of plasmid gene copies per genome were identical for all plasmids electroporated in this experiment (Fig. 4) \((F_{(4,10)} = 0.73, P = 0.589)\). This finding enables a comparison of the relative promoter strengths of the Ig-A and Ig-B regions used in the study. It can thus be concluded from these data that the Ig-B fragment, representing sequence upstream of the *ef-*1α orf-B in the *ef-*1α locus, is able to promote the highest level of luciferase activity when placed in the 5′ → 3′ orientation \((F_{(3,8)} = 43.57, P = 0.0001 \text{ at } 8 \text{ h})\), indicating that this fragment contains a relatively strong promoter. The Ig-A fragment cloned in the 5′ → 3′ orientation also promoted the expression of significantly higher luciferase activities compared to the control where the orientation of the sequence was reversed \((F_{(3,8)} = 18.33, P = 0.001 \text{ at } 8 \text{ h})\), and thus it also contains a promoter. Therefore, significant levels of luciferase expression were consistently obtained from transfected parasites containing the Ig-A and Ig-B fragments when cloned in the correct 5′ → 3′ orientation suggesting that the *ef-*1α IG region contains two independent promoters. The data indicates that the “Ig-B” region possesses higher promoter activity than the “Ig-A” region. However, this finding should not be interpreted as an indication of the actual strength of the “native” *ef-*1α-A and *ef-*1α-B promoters as they are arranged in the locus. True promoter strength should be estimated using full size IG regions cloned in the appropriate configuration relative to the target orf. Even if this is the case, this experimental approach, based on promoter regions cloned in transiently transfected plasmids, would not allow estimating the possible regulatory role of distantly located or “trans” enhancers, as well as the activity of other regulatory elements such as epigenetic factors. In addition, it is also possible that the promoter activity can be distinctly regulated in different stages of the life-cycle of the parasite, and the data discussed here may be applicable only to intra erythrocyte parasites developing in in vitro cultures. Therefore, it is likely that the absolute strength of these promoters as well as the study of its regulations could not be properly assessed using a limited transient

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**Fig. 5**

**a** Heterologous function of the *B. bigemina* IG promoters. Functional promoter analysis of the *B. bigemina* IG regions in *B. bovis* transfected parasites. The Y axis represents the PPE, and the Z axis represents RLU. The X axis represents the different plasmid constructs (Ig-A, Ig-A Rev, Ig-B, Ig-B Rev, 4-35-ef-luc, Pr+, pBluescript) transfected and the control (4-35-ef-luc not transfected) at the time of the measurements performed at different hours post-electroporation. The black bars represent the PPE ± standard deviation (SD). The grey line represents relative luciferase units (RLU).

**b** Schematic representation of the plasmid constructs used in the transient transfections and the RLU obtained 24 h after electroporation. Asterisks represent statistically significant differences detected using the t-test \((P < 0.05)\)
transfection approach such as the one used in this study. In summary, both regions tested seem to have different promoter strengths, despite conservation of the inverted repeats, but sequence divergence upstream these regions may account for differences in promoter strengths. Despite of these considerations, the data allows for the selection of Ig-B as a relatively strong promoter to be used in future stable transfection experiments using in vitro cultivated parasites.

**Heterologous promoter activities of the *B. bigemina* and *B. bovis* ef-1α promoters**

We then tested whether a promoter previously identified in the ef-1α IG region of *B. bovis* is also functional in *B. bigemina* and conversely, whether the *B. bigemina* ef-1α promoters described above are also functional in *B. bovis*. Luciferase activities in *B. bovis* transfected with plasmid 4–35 [5], containing the *B. bovis* ef-1α promoter for the ef-1α B gene (Fig. 5a) peaked at 24 h in all cases (Fig. 5a). After electroporation of the plasmids containing the *B. bigemina* ef-1α A and B promoters respectively into *B. bovis* parasites and, consistent with the data shown in Fig. 5a, we measured luciferase activities at its 24 h peak (Fig. 5b) (*F*_{(5,12)} = 12.03, *P* = 0.0001). The results of the comparative luciferase activities are shown in Fig. 5b. Analysis of growth rates performed at 8 and 24 h post-electroporation showed undistinguishable growth rates for all *B. bovis* transfected parasites (*F*_{(6,14)} = 0.92, *P* = 0.510 at 8 h and *F*_{(6,14)} = 2.07, *P* = 0.123 at 24 h), suggesting that parasite viability is equally affected by the procedure regardless of the transfection plasmid used in the electroporation (Additional file 1; Figure S1b). In addition, qPCR performed as described above demonstrates identical efficiencies for transfection regardless of the plasmid used (Fig. 6) (*F*_{(4,10)} = 1.27, *P* = 0.345), thus allowing comparisons of luciferase activities among the distinct promoters and estimating their relative strength under the conditions tested.

The *B. bovis* ef-1α promoter in Ig-B is able to promote expression of the luciferase gene in *B. bigemina* parasites at relatively high levels. This can be due to higher transfection efficiencies for the transfection of the plasmid into *B. bigemina*, to higher affinity of the *B. bovis* promoter for *B. bigemina* transcription factors, or alternatively, the lack of proper regulatory signals that can be involved in the regulation of the activity of the ef-1α promoter in *B. bigemina*. However, data in Fig. 6 demonstrates that all plasmids were transfected into the parasite genome with identical efficiencies, thus precluding the first possibility. Regardless of the possible explanations for the observed increased efficiency, *B. bovis* promoter is functional in *B. bigemina* parasites. Conversely, the *B. bigemina* promoter is also functional in *B. bovis* parasites. Recent work performed in *B. bovis* suggested the need for the use of heterologous promoters to increase the efficiency of the targeting of transfected genes by homologous recombination in this parasite [21]. Data obtained in this study confirms the feasibility for the use of heterologous promoters that can be functional among these two parasites. This will allow the improvement of *B. bovis* transfection-based knock-out (KO) and gene editing technologies, and the development of currently unavailable transfection KO systems for the functional gene characterization in *B. bigemina*.

**Conclusions**

In summary, a *B. bigemina* transient transfection system able to transfer exogenous DNA into both *B. bigemina* and *B. bovis* parasites was developed. The locus of the *B. bigemina* Puerto Rico strain elongation factor-1α was identified, characterized and the transcriptional activity of ef-1α promoters tested. This study demonstrates the existence of two distinct promoters with interchangeable homologous and heterologous promoter function in *B. bigemina* and *B. bovis*. The observation of interspecies promoter activity, described for the first time in *Babesia* species, is an important step for future stable transfection construct design and for the production of vaccines based on transfected parasites for *B. bigemina* and *B. bovis*.

**Additional file**

**Additional file 1:** Figure S1. a In vitro growth curve of *B. bigemina* parasites. The Y axis represents the PPE and X axis represents the different times points of measurement in hours. The transfected plasmid constructs are represented in lines Ig-A (blue diamond), Ig-A Rev (red square), Ig-B (green triangle), Ig-B Rev (purple x), 4-35-ef-luc (light blue asterisk), Pr- (orange circle) pBluescript (dark blue bar). b In vitro growth curve of *B. bovis* parasites. The Y axis represents the PPE and X axis represents the different times points of measurement in hours. The transfected construct plasmid are represented in lines Ig-A (blue diamond), Ig-A Rev (red square),
Abbreviations

bp: Base pair; ccpp-3: Limulus coagulation factor C domain protein-3; ef-1α: Elongation factor 1-alpha; gDNA: genomic DNA; IG: Intergenic; IR: Inverted repeats; iRBC: Infected red blood cells; KO: Knockout; luc: Luciferase; msa-1: Merozoites surface protein-1; orfs: Open reading frames; PCR: Polymerase chain reaction; pDNA: plasmid DNA; PPE: Percentage of parasitized erythrocytes; qPCR: quantitative real-time PCR; RACE: Rapid amplification of cDNA ends; RLU: Relative luciferase unit; SD: Standard deviation; TBDs: Tick and tick-borne diseases; UTR: Untranslated region

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Availability of data and materials

All data are disclosed as figures in the main document.

Authors’ contributions

MGS and CES designed the study and analyzed the results of experiments. CES wrote the manuscript. All authors read and approved the final findings in collaboration with MGS, maintain cultures, measured parasitemia and comparative analysis of apicomplexan hemoparasitic diseases to world animal health. Vet Parasitol. 1995;57(1-3):19–75.

References

1. Uilenberg G. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. Vet Parasitol. 1995;57(1-3):19–75.
2. Vial HJ, GorencFirst A. Chemotherapy against babesiosis. Vet Parasitol. 2006;138(1-2):147–60.
3. Suarez CE, Noël S. Emerging perspectives in the research of bovine babesiosis and anaplasmosis. Vet Parasitol. 2011;180(1-2):109–25.
4. Clark IA, Jacobson LS. Do babesiosis and malaria share a common disease process? Ann Trop Med Parasitol. 1998;92(4):483–8.
5. Suarez CE, McElwain TF. Transient transfection of purified Babesia bovis merozoites. Exp Parasitol. 2008;118(4):498–504.
6. Brayton KA, Lau AO, Herndon DR, Hännick L, Kappmeyer LS, Berens SJ, et al. Genome sequence of Babesia bovis and comparative analysis of apicomplexan hemoproteostasis. PLoS Pathog. 2007;3(10):1401–13.
7. Fujii K, Yokoyama N, Takabatake N, Suzuki H, Xuan XN, Igarashi I. Transient transfection of a green fluorescent protein gene in Babesia bovis. J Protozool Res. 2003;12:10–4.
8. Callow LL. Babesia bigemina in ticks grown on non-bovine hosts and its transmission to these hosts. Parasitology. 1966;55:375–81.
9. Graham OH, Hourlign J. Eradication programs for the arthropod parasites of livestock. J Med Entomol. 1977;13(6):629–58.
10. Buscher G. The infection of various tick species with Babesia bigemina, its transmission and identification. Parasitol Res. 1988;74(4):324–30.
11. Morzaria SP, Young AS, Hudson EB. Babesia bigemina in Kenya: experimental transmission by Boophilus decoloratus and the production of tick-derived stabiles. Parasitology. 1977;74(3):291–8.
12. Suarez CE, Norimine J, Lacy P, McElwain TF. Characterization and gene expression of Babesia bovis elongation factor-1α. Int J Parasitol. 2006;36(8):965–73.
13. Laughery JM, Knowles DP, Schneider DA, Bastos RG, McElwain TF, Suarez CE. Targeted surface expression of an exogenous antigen in stably transfected Babesia bovis. PLoS ONE. 2014;9(5):e97890.
14. Vidotto O, McElwain TF, Machado RZ, Pereyman LE, Suarez CE, Palmer GH. Babesia bigemina identification of B cell epitopes associated with parasitized erythrocytes. Exp Parasitol. 1995;81(4):491–500.
15. Rodriguez SD, Buening GM, Green TJ, Carson CA. Cloning of Babesia bovis msa-1 and comparison with Babesia bigemina msa-1. Parasitology. 1993;106(2):199–211.
16. Vega CA, Buening GM, Green TJ, Carson CA. In vitro cultivation of Babesia bigemina. Am. J Vet Res. 1985;46(2):416–20.
17. Levy MG, Ristic M. Babesia bovis: continuous cultivation in a microaerophilous stationary phase culture. Science. 1982;207(4436):1218–20.
18. Suarez CE, Palmer GH, Lefort C, Florin-Christensen M, Crabb B, McElwain TF. Intergenic regions in the hypoxia associated protein-1 (hp-1) locus promote exogenous gene expression in Babesia bovis. Int J Parasitol. 2004;34(10):1177–84.
19. Bastos RG, Ueti MW, Guerrero FD, Knowles DP, Scoles GA. Silencing of a putative immunophillin gene in the cattle tick Rhipicephalus (Boophilus) microplus increases the infection rate of Babesia bovis in larval progeny. Parasit Vectors. 2009;2(1):57.
20. Bastos RG, Suarez CE, Laughery JM, Johnson WC, Ueti MW, Knowles DP. Differential expression of three members of the multidomain adhesion CCP family in Babesia bigemina, Babesia bovis and Theileria equi. PLoS One. 2013;8(7):e67765.
21. Suarez CE, Johnson WC, Herndon DR, Laughery JM, Davis WC. Integration of a transfected gene into the genome of Babesia bovis occurs by legitimate homologous recombination mechanisms. Mol Biochem Parasitol. 2015;202(2):23–8.